Characterization of the Molecular Functions of Armadillo, Legless and Pygopus in Nuclear Wingless/Wnt Signalling

---

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Raymond Hoffmans

aus den

Niederlanden

Promotionskomitee

Prof. Dr. Konrad Basler (Vorsitz und Leitung der Dissertation)

Prof. Dr. Ernst Hafen

Zürich 2006

Contents

Summary

The Wingless (Wg)/Wnt signalling pathway controls fundamental processes during animal development. Deregulation of the Wg/Wnt pathway has been causally linked to several different forms of cancer, most notably to colorectal cancer. The key effector protein of the Wg/Wnt pathway is β-catenin which in the absence of the Wnt signal is bound and phosphorylated by its destruction complex consisting of Adenomatous Polyposis Coli (APC), Axin, GSK-3β and Casein Kinase Iα. Phosphorylated β-catenin is then marked for ubiquitination leading to proteasomal degradation. In the presence of the Wnt ligand, β-catenin is stabilized and can interact with transcription factors of the Lef/TCF family. β-catenin can further bind with CBP/p300, Brg-1 and Parafibromin, proteins that have been implicated in transcription. Originally, β-catenin was discovered for its role in cell adhesion. As part of the adherens junctions β-catenin binds with α-catenin and E-cadherin. Loss of adherens junctions has been linked with tumor metastasis.

Several years ago our lab identified two new proteins of the Wg/Wnt signalling cascade; they were named Legless (Lgs) and Pygopus (Pygo). On the basis of *in vivo* and biochemical experiments a model was proposed for nuclear Wg/Wnt signalling. In this model β-catenin binds Lef/TCF and the adapter protein Lgs which in turn recruits Pygo. In this quaternary complex Pygo would serve as a transcriptional activator. When I joined the lab we wanted to investigate if the Lgs-β-catenin interaction could be a valid target for therapeutic intervention in β-catenin dependent cancers. By using the yeast two-hybrid system I found that the β-catenin interaction with Lgs critically depends on two amino acids namely, D162 and D164. These acidic amino acids are specifically required for Lgs binding but not for TCF, E-cadherin or APC binding. I further showed that the Lgs interaction to both endogenous as well as constitutively active Armadillo (Arm, fly homolog of β-catenin) is necessary for Wg signalling activity *in vivo* in *Drosophila*. In a mammalian tissue culture system, Wnt signalling also depended on Lgs binding to β-catenin. These results suggest that Lgs-β-catenin interaction site can be disrupted specifically without interfering with the binding of β-catenin to the known tumor suppressor genes APC and E-cadherin. Disrupting the Lgs-β-catenin interaction has severe consequences for Wg/Wnt signalling but does not affect the adherens junctions and hence is an attractive drug target for therapeutic intervention.

Thus far we considered Lgs and Pygo as transcriptional coactivators of the pathway. This view was challenged by the publication of a model where Lgs and Pygo function as a nuclear anchor for β-catenin (Townsley et al., 2004). Together with Reto Städeli, another PhD student in the lab, we devised three different experiments in order to distinguish between the two models. In the first experiment we looked at the signalling and localization of constitutively active Arm/β-catenin *in vivo* in *Drosophila* and in mammalian tissue culture cells. We compared constitutively active Arm/β-catenin wildtype (wt) with a mutant (D164A) for Lgs binding. Both forms show a similar localization, but D164A has a severely reduced

signalling output compared to wt. In the second experiment I tested if one can bypass the need for Pygo. If the main function of Lgs would be to link β-catenin to the constitutively nuclear 'anchor' Pygo then Lgs with a nuclear localization signal should function independently of Pygo. However, constitutively nuclear Lgs still depended on Pygo binding for its function. In the third experiment Reto introduced a single amino acid substitution in Pygo. Although this mutant shows the same localization, expression level and binding strength to Lgs as wt Pygo, it resulted in severely reduced transcription of a Wg target gene. The results of these experiments argue strongly for a function of Lgs and Pygo as essential transcriptional coactivators to Arm/β-catenin.

In mammals there are two orthologs of Lgs, namely BCL9 and BCL9.2. The former was cloned in our lab after the discovery of Lgs in *Drosophila*. The latter was cloned recently by two groups (Adachi et al., 2004; Brembeck et al., 2004). One of them reported that BCL9.2 binding to β-catenin depends on tyrosine 142 of β-catenin (Brembeck et al., 2004). Since I identified the amino acids D162 and D164 of β-catenin to be essential for BCL9 and Lgs binding, I wanted to test if a Y142A mutant had a similar effect on BCL9 binding and if a D164A mutant could still bind BCL9.2. In a yeast two-hybrid assay, I found that the β-catenin-Y142 is essential for α-catenin binding but surprisingly not for BCL9.2 (nor BCL9) binding. However, BCL9.2 was also dependent on D164 for binding to β-catenin. Y142 is not necessary in both endogenous as well as constitutively active Arm for Wg signalling activity in *Drosophila*. In a mammalian tissue culture system, β-catenin signalling activity also did not depend on Y142. Furthermore it was reported that BCL9.2 transcriptional activation function does not depend of Pygo binding (Adachi et al., 2004; Brembeck et al., 2004). I demonstrated that BCL9.2 can functionally replace Lgs both in tissue culture and *in vivo* in *Drosophila* and is dependent on Pygo binding.

One of the most important questions that still needs further clarification is the function of Lgs and Pygo in Wnt signalling in vertebrates. I received from the Aguet lab (ISREC, Lausanne) Pygo double knock-out (KO) mouse embryonic fibroblasts (MEFs). Preliminary results from signalling assays show that the KO MEFs have lower β-catenin signalling activity then wt MEFs. The lower activity is caused by the lack of Pygo since adding Pygo to KO MEFs results in higher signalling activity.

Lgs binds β-catenin at a spatially different location then the proteins CBP/p300, Brg-1 and Parafibromin. The latter three bind β-catenin at the C-terminal half of the protein whereas Lgs binds more N-terminally. All these proteins have been implicated as transcriptional coactivators of β-catenin. In my most recent project I'm trying to decipher what the contributions are of Lgs binding versus binding of the other activators to β-catenin on Wnt signalling output. For this I compared activity of the D164A mutant with a mutant that has a deleted C-terminus (ΔC), which most likely is compromised in binding of CBP/p300 and Parafibromin. In *Drosophila* tissue culture cells and *in vivo*, the C-terminus contributes less than Lgs binding to signalling. However, in mammalian tissue culture cells the C-terminus is more important then Lgs binding for activity. The next step is to investigate the functional

importance of the D164A mutant versus the ΔC mutant in a vertebrate model organism. Therefore we started the generation of embryonic stem cells where the endogenous β-catenin gene is replaced by a mutant one. In total, three different knock-in cell and mouse lines will be created containing either β-catenin-D164A, β-catenin-ΔC or β-catenin-D164A-ΔC. The characterization of these cell lines and mice will represent an important step forward in the understanding of nuclear Wnt signalling.

Zusammenfassung

Der Wingless (Wg)/Wnt Signalweg kontrolliert grundlegende Abläufe während der Tierentwicklung. Deregulierung der Wg/Wnt Transduktion wird mit verschiedenen Formen von Krebs in Verbindung gebracht, am beachtenswertesten davon ist der Dickdarmkrebs. Das Schlüsselprotein der Wg/Wnt Signalkette ist β-catenin, welches in Abwesenheit des Wnt Signals gebunden und phosphoryliert wird durch den Zerstörungskomplex, zusammengesetzt aus den Proteinen Adenomatous Polyposis Coli (APC), Axin, GSK-3β und Casein Kinase Iα. Phosphoryliertes β-catenin wird dann markiert für Ubiquitinierung und proteolytischen Abbau. In der Anwesenheit des Wnt Signals wird β-catenin stabilisiert und kann mit Transkriptionsfaktoren der Lef/TCF Proteinfamilie binden. β-catenin kann ebenfalls binden mit CBP/p300, Brg-1 und Parafibromin. Diese Proteine sind alle beteiligt and der Transkription. Ursprünglich wurde β-catenin entdeckt durch seine Rolle in der Zell-Zell-Adhäsion. Als Teil der Adherens Junctions bindet β-catenin mit α-catenin und E-cadherin. Verlust von Zell-Zell-Kontakten kann Ursache für Metastasenbildung sein.

Vor einigen Jahren hat unser Labor zwei neue Proteine der Wg/Wnt Signalkette identifiziert und nannte diese Legless (Lgs) und Pygopus (Pygo). Durch *in vivo* und biochemische Experimente wurde ein Modell vorgeschlagen für Wg/Wnt Signaltransduktion im Zellkern. In diesem Modell bindet β-catenin mit Lef/TCF und mit dem Adaptorprotein Lgs, welches Pygo bindet. In diesem Komplex würde Pygo in der Transkriptionsaktivierung funktionieren. Zu Beginn meiner Arbeit wollten wir untersuchen, ob die Lgs-β-catenin-Interaktion ein mögliches Ziel wäre, um β-catenin-abhängigen Krebs therapeutisch zu bekämpfen. Durch den Gebrauch eines Yeast two-hybrid Systems fand ich, dass die β-catenin-Bindung zu Lgs abhängig ist von zwei Aminosäuren, D162 und D164. Diese Aminosäuren sind notwendig für die Lgs-Bindung, aber nicht für die TCF-, E-cadherin- oder APC-Bindung. Die Lgs-Interaktion mit Armadillo (Arm, *Drosohila* Homolog von β-catenin) ist extrem wichtig für die Wg Signalweg *in vivo* in *Drosophila*. Auch in einem Säugetier-Zellkultur-System ist die Lgs-Bindung zu β-catenin wichtig für die Wnt Signalkette. Die Resultate weisen darauf hin, dass die Lgs-Bindung zu β-catenin spezifisch unterbunden werden kann, ohne die Interaktion von β-catenin zu den Tumorsuppressor-Proteinen APC und E-cadherin zu stören. Die Zerstörung der Lgs-β-catenin-Bindung hat grosse Folgen für den Wg/Wnt Signalweg, aber nicht für Zell-Zell-Kontakte und deshalb ist diese Interaktion medizinisch höchst interessant.

Bisher sahen wir Lgs und Pygo als transkriptions-aktivierende Moleküle der Wg/Wnt Signalkette. Diese Sichtweise wurde herausgefordert durch eine Publikation, in deren Modell Lgs und Pygo als nukleäre Anker für b-catenin funktionieren (Townsley et al., 2004). Zusammen mit Reto Städeli, einem anderen PhD Studenten im Labor, habe ich drei Experimente entworfen, um zwischen diesen zwei Modellen unterscheiden zu können. Im

ersten Experiment haben wir die Signalstärke und die Lokalisierung von aktiviertem Arm/β-catenin *in vivo* in *Drosophila* und in einem Säugetier-Zellkultur-System beobachtet. Wir haben aktiviertes wildtyp Arm/β-catenin (wt) verglichen mit der mutanten Form (D164A), welche Lgs nicht binden kann. Beide Formen zeigten die gleiche Lokalisation, aber D164A hatte einen stark reduzierten Signal-Output im Vergleich zum wt. Im zweiten Experiment habe ich getestet, ob die Signalkette ohne Pygo funktioniert. Wenn es die Hauptfunktion von Lgs wäre, β-catenin mit dem nukleären Anker Pygo zu verbinden, dann sollte Lgs mit einem Kern-Lokalisations-Signal (NLS-Lgs) unabhängig von Pygo sein. Ein konstitutiv nukleäres Lgs (NLS-Lgs) ist jedoch noch immer abhängig von Pygo für seine Funktion. Im dritten Experiment hat Reto eine einzige Aminosäure in Pygo ersetzt. Obwohl diese Mutante im Vergleich zu Pygo wt die nahezu gleiche Lokalisation und Expression aufweist und gleich stark an Lgs bindet, zeigt die Mutante stark reduzierte Transkriptionsaktivierung eines Wg Zielgens. Die Resultate dieser Experimente weisen stark auf eine Funktion von Lgs und Pygo als transkriptionsaktivierende Moleküle für Arm/β-catenin hin.

In Säugetieren gibt es zwei Orthologe von Lgs, nämlich BCL9 und BCL9.2. BCL9 wurde in unserem Labor kloniert, nach der Identifizierung von Lgs in *Drosophila*. BCL9.2 wurde von zwei verschiedenen Gruppen (Adachi et al., 2004; Brembeck et al., 2004) kloniert. Eine berichtete, dass die BCL9.2-Bindung zu β-catenin abhängig ist von Tyrosine 142 in β-catenin (Brembeck et al., 2004). In meinen Experimenten waren die Aminosäuren D162 und D164 in β-catenin erforderlich für die BCL9- und Lgs-Bindung. Deshalb wollte ich testen, ob eine Y142A-Mutation in Arm denselben Effekt auf die BCL9-Bindung habe und ob eine D164A-mutante Form von Arm noch immer BCL9.2 binden könne. Ein Yeast two-hybrid-Experiment zeigte, dass β-catenin-Y142 notwendig ist für die α-catenin-Bindung, aber überraschenderweise nicht für die BCL9.2- und BCL9-Bindung. BCL9.2 ist jedoch abhängig von D164 für seine Bindung zu β-catenin. Arm-Y142 ist nicht wichtig für den Wg Signalweg *in vivo* in *Drosophila*. Auch in einem Säugetier-Zellkultur-System ist der β-catenin Signal-Output nicht abhängig von Y142. Ausserdem sollte die Transkriptionsaktivierungs-Funktion von BCL9.2 nicht von der Interaktion mit Pygo abhängen (Adachi et al., 2004; Brembeck et al., 2004). Ich konnte jedoch in Zellkultur und *in vivo* in *Drosophila* zeigen, dass BCL9.2 das funktionelle Homolog von Lgs ist und dass dessen Funktion ebenfalls abhängt von der Interaktion mit Pygo.

Eine der wichtigsten Fragen, die noch aufgeklärt werden muss, ist die Funktion von Lgs und Pygo in der Wnt Signalkette in Wirbeltieren. Vom Aguet Labor (ISREC, Lausanne) habe ich pygo-doppel-knock-out (KO) embryonale Mausfibroblasten (MEFs) bekommen. Die ersten Resultate von Signal Output Experimenten zeigten, dass die KO MEFs niedrigere β-catenin Transkriptionsaktivierung hatten als wt MEFs. Die gesenkte Aktivität wurde verursacht durch die Abwesenheit von Pygo. Zugabe von Pygo zu den KO MEFs führt zu einer höheren Aktivität.

Lgs bindet β-catenin an einem anderen Ort als die Proteine CBP/p300, Brg-1 und Parafibromin, welche β-catenin am C-terminalen Teil binden. Lgs bindet am N-terminalen

Teil. Jedes von diesen Molekülen ist beteiligt an der Transkriptionsaktivierung von β-catenin. In meinem letzten Projekt versuche ich herauszufinden, was der Anteil der Lgs-Bindung verglichen mit der Bindung der anderen Moleküle zu β-catenin für die Wnt Signalkette ist. Deshalb vergleiche ich die Aktivität der D164A-Mutanten mit einer Mutante, welcher der C-terminus fehlt (ΔC). Diese Mutante kann höchst wahrscheinlich CBP/p300 und Parafibromin nicht binden. In *Drosophila* (Zellkultur und *in vivo*) trägt der C-terminus von Arm weniger zur Wg Signalkette bei als die Lgs-Bindung. In einer Säugetier-Zellkultur hingegen trägt der C-terminus mehr bei als die Lgs-Bindung. Der nächste Schritt ist die Untersuchung des Anteils einer Lgs-Bindemutante von β-catenin verglichen mit einer C-terminalen Deletion (ΔC) in einem Wirbeltier Modellorganismus. Deshalb haben wir mit der Herstellung von embryonalen Stammzellen begonnen, in welchen das endogene β-catenin-Gen ersetzt wird durch ein mutantes. Insgesamt werden drei verschiedene knock-in Zell- und Maus-Linien erzeugt mit β-catenin-D164A, β-catenin-ΔC oder β-catenin-D164A-ΔC. Die Charakterisierung dieser Zelllinien und Mäuse wird ein grosser Schritt sein hin zum besseren Verständnis des Wnt Signalweges im Zellkern.

1. Introduction

1.1 Transcription under nuclear Arm's control

Reto Städeli[1], Raymond Hoffmans[1] and Konrad Basler

Accepted in Current Biology

[1] These authors contributed equally to this work and should be considered co-first authors.

Abstract

The Wingless/Wnt pathway controls cell fates during animal development and regulates tissue homeostasis as well as stem cell number and differentiation in epithelia. Deregulation of Wnt signaling has been associated with cancer in humans. In the nucleus, the Wingless/Wnt signal is transmitted via the key effector protein Armadillo/β-catenin. The recent identification and functional analysis of novel Armadillo/β-catenin interaction partners provide new and exciting insights into the highly complex mechanism of Wingless/Wnt target gene activation.

Signaling molecules of the Wingless (Wg)/Wnt family are secreted glycoproteins that control a diverse array of processes in both embryos and adults. The Wnt cascade has been implicated in the postembryonic regulation of stem cell number and differentiation of several adult stem cell systems (reviewed in [1, 2]). Moreover, the pathway has been causally linked to various diseases, most notably to cancer (reviewed in [3, 4]). In the absence of the Wg/Wnt signal, DNA-bound transcription factors of the T-cell factor (TCF) family of HMG-box proteins bind the transcriptional repressor proteins Groucho/TLE and CtBP. Upon activation of the pathway the key transducing component Armadillo/β-catenin becomes stabilized, enters the nucleus and heterodimerizes with TCFs to activate the expression of Wg/Wnt target genes. This co-activator function of Arm/β-catenin depends mainly on two arms: An N-terminal activating arm (NTAA) recruiting Legless and Pygopus, and a C-terminal activating arm (CTAA) binding to CBP, Brahma/Brg-1, MED12, and Hyrax/Parafibromin. Despite its therapeutic relevance, the mechanisms by which Arm/β-catenin employs these co-factors to control the transcription of target genes is only poorly understood. Here we review past and recent findings that relate to this problem and discuss how they can be integrated into a more complete picture of Wg/Wnt target gene activation.

The Armament of the Wg/Wnt Signaling System

Armadillo (Arm)/β-catenin fulfills two main functions in the cell: (i) as a component of the cadherin-based cell adhesion system it binds the transmembrane protein E-cadherin and regulates actin filament assembly via α-catenin (reviewed in [5]); and (ii) as a nuclear regulator of Wingless (Wg)/Wnt dependent gene expression it provides transcriptional activator functions to TCFs. The Arm/β-catenin protein consists of a central region that is made up of 12 imperfect armadillo repeats (R1-12) flanked by an N- and a C-terminal tail [6]. There are forms of Arm carrying mutations or deletions which interfere with the adhesion function, but not with its role in Wg/Wnt signaling, and vice versa, indicating that these two functions of Arm are independent and separable [7, 8]. Interestingly, the small nematode *C. elegans* has three different β-catenin proteins which possess separate functions dedicated either to adhesion (HMP-2) or to Wnt signaling (WRM-1 and BAR-1) [9]. In the absence of the Wg/Wnt signal, cytosolic Arm/β-catenin is constantly phosphorylated by the action of a so-called ‘degradation complex’ consisting of the Adenomatous polyposis coli (APC) protein,

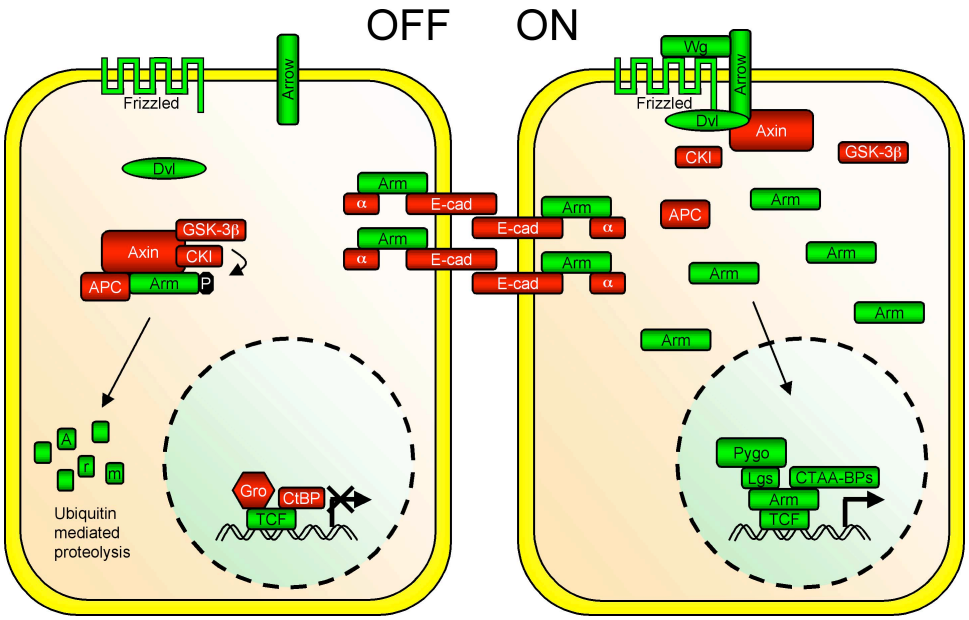

Figure 1: Simplified overview of the Wg/Wnt signaling pathway

The cell to the left shows the OFF state of the Wg/Wnt pathway. In the absence of the Wnt signal, Armadillo (Arm)/β-catenin protein levels are downregulated by a complex containing Adenomatous polyposis coli (APC) protein, Axin, casein kinase I (CKI), and glycogen synthase kinase-3 (GSK-3β). In addition, the co-repressors Groucho (Gro)/TLE and C-terminal binding protein (CtBP) are bound to T-cell factor (TCF). The cell to the right represents the ON state of the pathway. The Wg/Wnt ligand binds Frizzled and its co-receptor Arrow/LRP (LDL-receptor-related protein). Axin is bound by Dishevelled (Dvl) and Arrow, thereby disrupting the 'degradation complex'. Arm/β-catenin accumulates in the cytoplasm, enters the nucleus and displaces Gro from TCF. For transcription of target genes Arm/β-catenin interacts with Legless (Lgs), which binds Pygopus (Pygo), and with C-terminal activating arm binding proteins (CTAA-BPs) such as CREB-binding protein (CBP)/p300, Hyrax/Parafibromin, TATA Binding Protein (TBP), MED12, and Brahma/Brg-1 (Brahma-related gene-1). As part of adherens junctions, Arm binds the transmembrane protein E-cadherin (E-cad) and the cytoplasmic protein α-catenin (α). Negatively acting components of the pathway are colored in red with white letters, while positive components are shown in green with black letters.

Axin, and the kinases casein kinase I (CKI) and Shaggy/Zeste white-3 (GSK-3β) and hence rapidly degraded via the ubiquitin/proteasome pathway (reviewed in [10]). Interaction of the Wg/Wnt ligand with its receptors Frizzled and Arrow/LRP (LDL-receptor-related protein) blocks the degradation complex and leads to the stabilization of Arm/β-catenin. Such Arm/β-catenin protein accumulates in the cytoplasm and can enter the nucleus, where it acts as a co-activator of TCFs [11-16] (Figure 1). Artificially preventing Arm from entering the nucleus blocks Wnt signaling [17], while forcing it into the nucleus can have the opposite effect [18-20]. Therefore, the signaling activity of Arm/β-catenin is largely controlled by the size of its nuclear pool, which depends on the cytoplasmic levels of β-catenin as well as on the import into and export out of the nucleus.

Arm's Import and Export

The business of Arm's import and export is rather nebulous and somewhat controversial. Below we summarize what has been reported. β-catenin appears to be imported into the nucleus in a NLS- and importin/karyopherin-independent manner by directly interacting with nuclear pore components [21, 22]. Addition of cytosol inhibited nuclear import of β-catenin [21, 22], indicative of the presence of cytosolic retention factors. Indeed, it was shown that

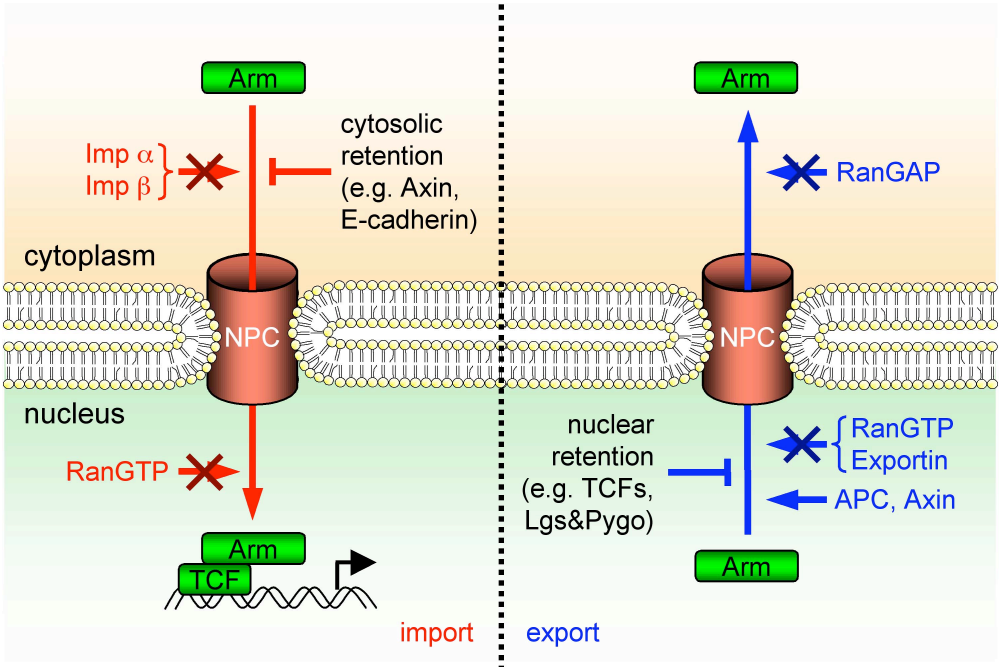

Figure 2: Nucleo-cytoplasmic shuttling of Arm/β-catenin

Left side: Nuclear import of Arm works independently of the carrier proteins Importin (Imp) α/β and is mediated by direct interaction of Arm with components of the nuclear pore complex (NPC). Arm's release into the nucleus does not depend on RanGTP. Nuclear import of Arm can be counteracted by cytosolic retention factors such as Axin and E-cadherin.

Right side: Nuclear export of Arm works in an Exportin-/RanGTP-independent manner, and no Ran GTPase activating protein (RanGAP) is required to release Arm on the cytosolic side. Proteins like APC and Axin, which can bind Arm and shuttle between the nucleus and the cytoplasm (Ran dependent), may facilitate nuclear export of Arm, whereas nuclear proteins that interact with Arm, like TCFs or Legless (together with Pygopus), might serve as nuclear retention factors and decrease the nuclear export rate of Arm.

Axin could play such a role in *Drosophila* [23]. Pangolin (Pan, dTCF), on the other hand, can function to keep Arm in the nucleus [23]. However, a mutant form of Arm, that is defective in binding Pan, still localizes to the nucleus [7, 15]. It has also been proposed that Legless (Lgs) in cooperation with Pygopus (Pygo) serves as a nuclear anchor for Arm [20]. However, Arm still localizes to the nucleus in *axin pygo* double-mutant clones, suggesting that Pygo acts downstream of Arm nuclear localization [24, 25]. These findings show the difficulty in designing experiments to clearly separate the process of nuclear import and retention of Arm/β-catenin. While the region comprising R10-C seems to be necessary and sufficient for the nuclear import [26], different regions of Arm/β-catenin that interact with binding partners such as the above mentioned dTCF and Lgs (together with Pygo) could serve as anchor points contributing to nuclear retention of Arm/β-catenin. To further increase complexity, Arm also has an intrinsic nuclear export activity [21, 22, 27], which has been mapped and shown to overlap with its 'import region' [26]. Furthermore, Axin [28] and APC [29-31] may facilitate nuclear export of Arm. However, a recent paper that investigated the nucleo-cytoplasmic shuttling of β-catenin and its relation to TCF4, BCL9 (B-cell lymphoma 9, Lgs homolog in mammals), APC, and Axin [32] demonstrated that these proteins do not accelerate the

import/export rate of β-catenin. They rather act in retaining β-catenin in the compartment in which they are localized. In summary, the intracellular localization of Arm/β-catenin represents a dynamic equilibrium of its intrinsic nuclear import and export activities as well as the availability and affinity of its binding partners (Figure 2).

Harm by nuclear Arm: Breaking the 'non-proliferation treaty'

Mutations that constitutively stabilize Arm/β-catenin can cause colorectal carcinomas and other forms of cancer. One recent view described such cancers as a 'stem cell disease' [33]. All cells in a normal colonic crypt are thought to be derived from epithelial stem cells located to the bottom of each crypt and maintained as stem cells by a Wnt signaling system [34]. Aberrant activation of the Wnt pathway leads to an expansion of this stem cell population into upper regions of the crypt. Instead of differentiating such cells assume stem cell-like replication properties, causing overproliferation and the accumulation of mutations, finally resulting in polyps and adenomatous lesions in the colon (Figure 3) (reviewed in [33, 35]). The best-studied example, Familial Adenomatous Polyposis (FAP), is in most cases caused by truncations in APC [36, 37]. Truncated APC can no longer fulfill its function in Arm/β-catenin degradation. As a consequence, Arm/β-catenin accumulates and enters the nucleus where it activates target genes implicated in cell proliferation (e.g. c-Myc [38] and gastrin [39]), inhibition of apoptosis (e.g. survivin [40]), and tumor progression (e.g. Laminin γ2 [41]) (Figure 3). The molecular basis of how such target genes exert their harmful effect in the various steps towards tumorigenesis is currently not well understood and will not be discussed further. However, especially the Arm/β-catenin targets implicated in cell proliferation would fit into the view of cancer as a 'stem cell disease'. How are these nuclear Arm/β-catenin targets activated?

Nuclear Arm races towards TCF and displaces Groucho/TLE

In the absence of nuclear Arm/β-catenin, Pan/TCF is bound to Groucho/TLE [42, 43] and CtBP (C-terminal binding protein) [44-47]. Groucho/TLE family proteins are general long-range transcriptional co-repressors, which have been shown to interact with histone deacetylases (HDAC) [48]. Reduction of Pan or Groucho in the *Drosophila* embryo results in partial suppression of *armadillo* and *wingless* mutant phenotypes, whereas overexpression of Pan enhances the phenotype of a weak *wingless* allele [43]. This suggests that, in the absence of Wg signaling, a Pan-Groucho complex acts as a repressor of Wg targets in the embryo. In contrast, loss of Pan in the *Drosophila* wing imaginal disc results in a reduction of Wg target gene expression, but does not cause a derepression of these genes outside the normal domain of expression. Loss of Groucho also does not result in derepression of Wg target genes [49]. Thus, Pangolin does not appear to function by default as a repressor in the wing imaginal disc in the absence of Wg signaling. Rather it seems that the repressor function of Pan can vary in different tissues. Nuclear Arm/β-catenin binds to the N-terminus of Pan/TCF and displaces Groucho/TLE by binding to a second, low-affinity binding site on TCF

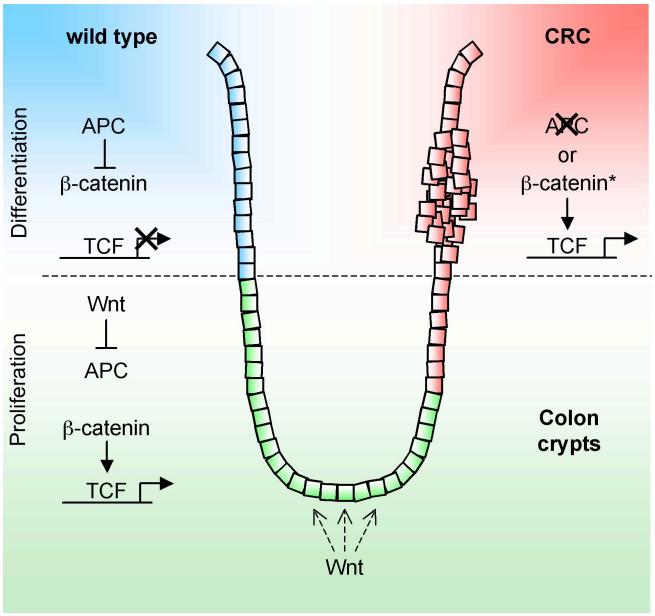

Figure 3: Model for colorectal cancer (CRC)

The left side of the crypt is wild type. A Wnt signal at the bottom of the crypt blocks β-catenin degradation, thereby activating target genes that maintain cell proliferation (green cells). Halfway up the crypts, β-catenin is downregulated and the progenitor crypt cells start to differentiate (blue cells). The right side of the crypt shows cells that have a mutation in either APC or β-catenin (red cells), which results in a constitutive activation of the target genes and prevents such cells from differentiating. The cells maintain their progenitor state and continuously proliferate. Adapted from [90].

located in the C-terminal half of the protein, which overlaps with the Groucho/TLE binding site [50]. Interestingly, neither the N- nor the C-terminus of β-catenin are necessary for this displacement, suggesting that it is – like TCF binding – accomplished by the central Arm repeat domain. CtBP proteins are general short-range transcriptional co-repressors that interact with HDACs (reviewed in [51]). TCF3 and TCF4 have been shown to bind CtBP *in vitro* and *in vivo* [44-47]. The binding of CtBP represses TCF3- and TCF4-mediated transcription [44-46] and this repression depends on HDAC activity [44]. To date it is not clear how Wnt signaling overcomes the repressor effect of CtBP on TCF3 and TCF4 and whether β-catenin plays an active role in this process.

After the release of the Grouche/TLE and CtBP repressor systems from Pan/TCF, nuclear Arm/β-catenin can fully develop its transcriptional activator potential.

Arm's Activating Arms

In the nucleus, Arm/β-catenin binds the DNA binding protein Pan/TCF/LEF through R3-10 [11-16, 52] (Figure 4). In addition to the Pan/TCF/LEF binding domain, there are two other domains which are important for Wg/Wnt signaling, namely R1 and R11-C [7]. It was shown recently that R1-4 are necessary and sufficient for Lgs binding [8, 53-55], and that R11-C are important for the binding of proteins such as CBP (CREB-binding protein)/p300, Hyrax/Parafibromin, TBP (TATA Binding Protein), MED12, and Brahma/Brg-1 (Brahma-related gene-1) [56-61]. Mutations in or deletions of these two domains result in Arm/β-catenin proteins with reduced signaling activity [7, 8, 15, 62, 63]. On the other hand, both domains have been shown to exhibit signaling activity on their own [15, 18, 54]. From here on

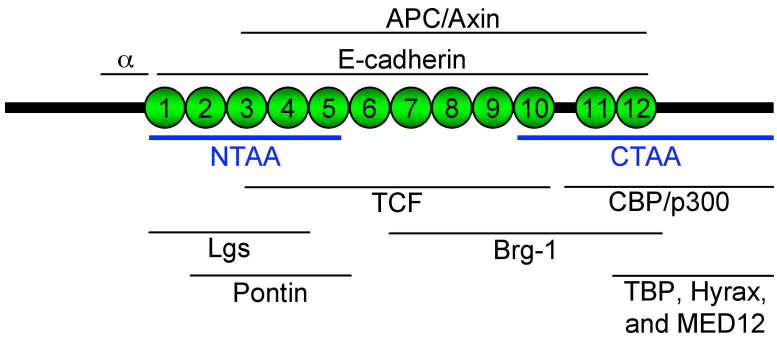

Figure 4: Schematic overview of what binds where on Arm/β-catenin

The Arm repeats are numbered 1–12. Blue lines show the N-terminal activating arm (NTAA) and the C-terminal activating arm (CTAA). The black lines represent binding domains of Arm/β-catenin interacting proteins. APC (Adenomatous polyposis coli), α (α-catenin), TCF (T-cell factor), Lgs (Legless), CBP (CREB-binding protein, CREB (cAMP response-element binding protein)), Brg-1 (Brahma-related gene-1), TBP (TATA binding protein), MED12 (Mediator subunit 12).

we refer to these two activating arms of Arm/β-catenin as 'NTAA' and 'CTAA' for N-terminal activating arm (R1-5) and C-terminal activating arm (R10-C), respectively.

An armada of nuclear Arm auxiliary factors

CTAA collaborators

β-catenin has several partners that bind the CTAA and contribute to transcriptional activation, five of which are briefly discussed here. (i) One of these proteins is TBP, which has been shown to bind β-catenin at R12-C [57]. Noteworthy, there are two more domains in β-catenin that bind TBP, namely the N-terminus and R2-4. So far, solid experimental data for the functional significance of the β-catenin–TBP interaction is lacking. (ii) Brg-1, a component of mammalian SWI/SNF and Rsc chromatin remodeling complexes, has been shown to bind Arm/β-catenin through R7-12. Overexpression of Brg-1 promotes transcriptional activation of TCF-responsive reporter genes. In *Drosophila*, Brahma genetically interacts with Arm [56]. (iii) p300 and the closely related CBP are co-activators that link proteins to the basal transcription machinery or alter chromatin structure through their intrinsic or associated histone acetyltransferase (HAT) activities. p300 and CBP bind R10-C of β-catenin and stimulate transcriptional activity [58, 59]. Interestingly, although the co-activators p300 and CBP are closely related, they do not always exert the same effects on promoters; for instance, they have opposite effects on the β-catenin-mediated expression of the *survivin* gene. The small molecule ICG-001, which specifically blocks the CBP-β-catenin interaction, but not the p300-β-catenin interaction, inhibits *survivin* gene expression. In the absence of ICG-001, CBP is associated with the *survivin* promoter. In the presence of ICG-001 there is less CBP at the promoter but more p300 instead, which in turn recruits repressive elements and results in a reduction of *survivin* transcription [64]. (iv) Recently, it has been shown that the human and Drosophila homologs of yeast Cdc73p (Parafibromin and Hyrax, respectively) are involved in Wg/Wnt signaling. Cdc73p is a component of the PAF1 complex, a conserved RNA

polymerase II interacting complex, which has been implicated in the regulation of transcriptional initiation and elongation (reviewed in [65]). Hyrax and Parafibromin bind to R12-C of Arm and β-catenin, respectively. Overexpression of these proteins results in an increase of Wg/Wnt pathway activity, while experimental reduction of their levels has the opposite effect [60]. (v) MED12, a component of the Mediator complex, has been found to interact with β-catenin R12-C [61]. The Mediator (MED) complex, first discovered in yeast, links transcriptional regulators to RNA polymerase II (Pol II) and general transcription factors [66-68]. Meanwhile, counterparts for nearly all yeast MED components have been discovered in mammals (reviewed in [69-71]). Both RNAi-mediated knock-down of MED12 as well as overexpression of the isolated β-catenin binding domain of MED12 in HeLa cells impaires β-catenin-dependent transactivation in response to Wnt signaling [61].

Beside the five co-activator partners, described above, that bind directly to the CTAA of β-catenin, it was recently shown that the TRRAP/TIP60, the ISW1, and the MLL1/MLL2 SET1-type complexes also selectively associate with the CTAA [72]. All three of these complexes are involved in histone modification and chromatin remodeling. It has not been resolved yet whether these complexes interact directly with β-catenin.

NTAA collaborators

Pontin/Tip49 and Reptin/Tip48 are two highly homologous proteins, which can form homo- and heterodimers. They have been shown to bind to β-catenin R2-5 and antagonistically affect β-catenin output. Reptin inhibits and Pontin enhances transcriptional activation of reporter genes. In *Drosophila*, mutations in the *pontin* and *reptin* genes exhibited opposite dominant effects on wing phenotypes in a genetic background sensitized for Arm signaling [73, 74]. Also in zebrafish Pontin and Reptin function antagonistically: the *liebeskummer (lik)* mutation encodes an activated Reptin protein, which promotes cardiac hyperplasia. This phenotype was enhanced by reduction of β-catenin or pontin expression in a heterozygous *lik* mutant [75]. In a chromatin immunoprecipitation experiment Pontin, Tip60, and TRRAP interacted with the promoter of a TCF-dependent gene, *ITF-2* (immunoglobulin transcription factor-2). Overexpression of a mutant inactive form of Pontin resulted in decreased acetylation of histones and reduction of *ITF-2* expression [76]. The domains of β-catenin, to which Pontin, Reptin and Lgs bind, are overlapping (R2-5 and R1-4). However, it is presently unknown whether they bind simultaneously or in a competitive manner.

Lgs and Pygo were discovered in genetic screens for modifiers of the Drosophila Wg pathway [24, 25, 53, 77]. Loss of *lgs* or *pygo* function results in a severe reduction of pathway output. Arm/β-catenin binds Lgs through R1-4 and the acidic amino acids D162 and D164 play a key role in this protein-protein interaction [8, 53]. Replacing wild-type Arm with Arm-D164A (which cannot bind Lgs) causes phenotypes that are very similar to those of *lgs* null mutants [8]. The predominantly nuclear localization of Lgs depends on the presence of Pygo [20]. It has been proposed that Lgs and Pygo function mainly to enhance the nuclear levels of

Arm [20, 78]. This notion was based on an experiment in which the cuticular phenotype of *lgs* and *pygo* mutant embryos was ameliorated by overexpressing a form of Arm with a nuclear localization signal. This experiment indeed showed that high amounts of nuclear Arm can activate dTCF targets in the absence of Lgs or Pygo, most likely by displacing Groucho/TLE from Pan and by recruiting the above-described activators via the CTAA. However, in a different set of experiments it was shown that a DNA-tethered and constitutively nuclear form of β-catenin can only very poorly activate reporter gene expression if it carries the D164A mutation, suggesting that BCL9 binding critically contributes to β-catenin's activator capacity [79]. Furthermore, constitutive nuclear targeting of Lgs does not bypass the requirement for Pygo in Wg signaling, indicating that Pygo must provide a function beyond ensuring the availability of Lgs and Arm/β-catenin in the nucleus of Wg-transducing cells [79]. These findings therefore argue that Arm depends on Lgs and Pygo primarily for its transcriptional output rather than for its nuclear import or retention. They also argue that in addition to the Arm/β-catenin CTAA, there is a Lgs-Pygo-dependent output from the NTAA important for Arm activity. In this NTAA function, Lgs appears to serve as an adaptor protein linking Pygo to Arm/β-catenin [53] while the N-terminal homology domain of Pygo (PygoNHD) is a critical mediator of this function, as it cannot be bypassed [54].

How to activate a nuclear Arm…

Transcriptional activation of target genes requires the recruitment of ATP-dependent chromatin remodeling enzymes and histone acetyltransferase (HAT) complexes to their promoters to 'prepare' the chromatin. There appears to be no obligate order of function for these complexes (reviewed in [80]). After chromatin preparation, RNA polymerase II is recruited and transcription initiated. In the case of the LEF-β-catenin complex it has been shown that R11-C of β-catenin is necessary for chromatin remodelling in vitro [81]. This region of β-catenin partially overlaps with binding sites for Brg-1 (R7-12) and p300/CBP (R10-C). These two proteins could be involved in the preparation of chromatin at Wg/Wnt target genes. In addition, components of the MLL/SET1-type chromatin modifying complexes associate with this domain of β-catenin and augment histone methyl transferase (HMT) activity and H3K4 trimethylation at the Wnt target gene c-Myc [72].

With the identification of MED12 as a β-catenin binding protein, there is now a link between β-catenin and Pol II: The MED complex can associate with Pol II to generate a stable complex sometimes called the Pol II holoenzyme (reviewed in [82, 83]). Yet another link between β-catenin and Pol II comes forward with the discovery of Hyrax/Parafibromin as an Arm/β-catenin interaction partner, which interestingly also binds to the CTAA and thus overlaps with the Brahma/Brg-1, CBP/p300, and MED12 binding sites. The PAF1 (polymerase associated factor) complex, which contains Parafibromin, has been found to associate with the nonphosphorylated and Ser2 and Ser5 phosphorylated forms of the Pol II large subunit [84]. Another study describes the tumor suppressor Parafibromin as a PAF1 complex- and Pol II-bound protein acting in transcription elongation and RNA processing [85].

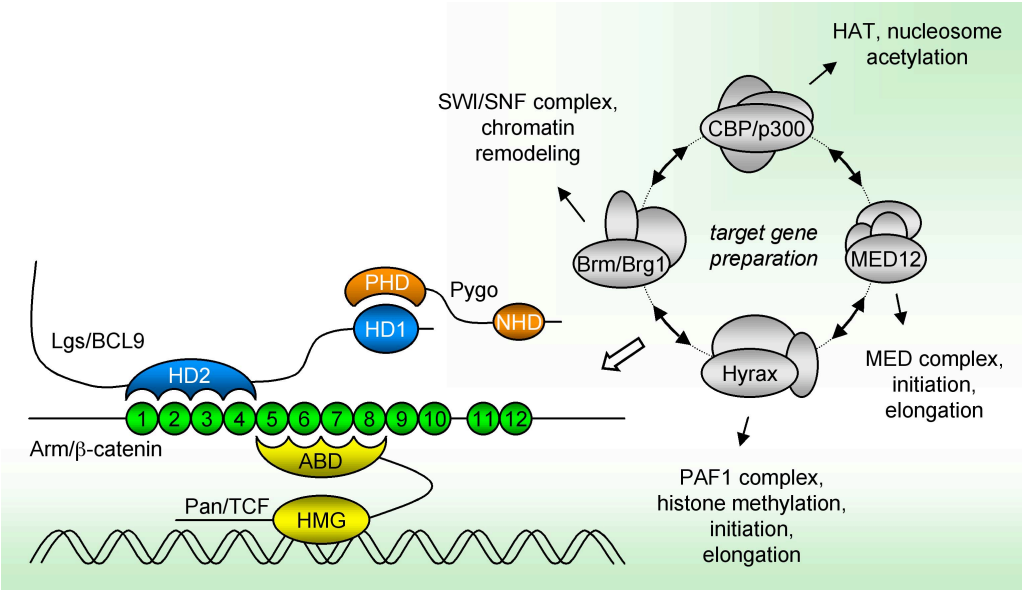

Figure 5: Model of nuclear Arm's control over transcription

Nuclear Arm is recruited to Wingless target genes by high mobility group (HMG) transcription factors of the TCF/LEF family, which all have an Armadillo-binding domain (ABD) at their very N-termini. Arm acts through two activating arms. (i) The N-terminal activating arm acts mainly through binding Legless (Lgs) at its homology domain 2 (HD2). Lgs, via its homology domain 1 (HD1), can recruit the co-activator Pygopus (Pygo) by binding its plant homology domain (PHD). (ii) The C-terminal activating arm can interact with protein complexes exhibiting histone acetyltransferase (HAT) or chromatin remodeling (SWI/SNF complex) activities. Other interaction partners are components that can recruit histone methyltransferases (e.g. MLL/SET1) or play important roles in transcription initiation and elongation (PAF1 complex, MED complex). Sequential or random recruitment of these factors and complexes, step-by-step, results in a more accessible chromatin structure and finally leads to transcription of the target gene.

As the PAF1 complex has been shown to physically interact with the SET1 complex [86], an attractive idea would describe the role of Hyrax/Parafabromin as a recruiting module not only for Pol II, but also for the MLL/SET1-type complexes.

Mosimann et al. [60] showed that there might be a Hyrax/Parafibromin-related crosstalk between the CTAA of β-catenin and Pygo. As described above, overexpression of Parafibromin leads to an increase in Wnt-reporter gene activity in tissue culture cells. However, this increase depends on Pygo as it can be abrogated by the siRNA-mediated reduction of Pygo. In other words, the activating function of Hyrax/Parafibromin in the Wg/Wnt pathway seems to depend on the recruitment of Pygo to β-catenin. These findings could be interpreted to implicate Pygo in stabilizing or exchanging trans-activating complexes that bind the Arm/β-catenin CTAA.

Taken together, a model can be composed (depicted in Figure 5) in which Wg/Wnt target gene activation might be a concerted, Pygo-assisted process, which dynamically coordinates the sequential action of transcriptional modulators at the central scaffold protein Arm/β-catenin.

… and how to disarm it

Although not much is currently known about how Arm/β-catenin-regulated genes are turned off, three processes are likely to be important: (i) disassembly of the Arm/β-catenin enhancer complex, (ii) reversion of the 'activating' histone modifications, and (iii) nuclear export and degradation of Arm/β-catenin. Recent work by Sierra et al. [72] raises the possibility that APC may be a key protein involved in all three of these processes. The association of full-length APC with the Wnt-dependent enhancer of the c-Myc gene correlated with the disassembly of the β-catenin enhancer complex, resulting in a rapid decrease of c-Myc mRNA levels. Sierra et al. [72] proposed that full-length APC may recruit CtBP and β-TrCP (β-Transducin repeats-containing protein) to the c-Myc enhancer. APC has previously been shown to directly interact with CtBP, both in vivo and in vitro [47]. LSD1 (Lysine Specific Demethylase 1), a component of CtBP complexes, has been implicated in reverting H3K4 mono- and di-methylation in vitro [87]. To our knowledge, factors that catalyze the removal of a methyl group from tri-methylated lysines (e.g. H3K4$(CH_3)_3$) have not yet been described. APC may further bind to Arm/β-catenin and remove it from TCF/LEF, which in turn is then free to recruit Groucho/TLE-1 and HDAC complexes, while APC might facilitate nuclear export of Arm/β-catenin (reviewed in [88, 89]) and promote its cytosolic degradation.

This scenario, in which a nuclear pool of APC counteracts Wnt signaling at multiple levels, still lacks definitive experimental confirmation and also raises some unanswered questions. For example, how is APC recruited to enhancers of TCF/LEF target genes, when Arm/β-catenin cannot interact simultaneously with TCF/LEF and APC due to overlapping binding sites? Does APC perhaps interact with other subunits of the Arm/β-catenin enhancer complex? Undoubtedly however, the mechanism by which APC counteracts transcription of Wg/Wnt targets will be key to understand how Arm and β-catenin undergo nuclear disarmament.

The future of nuclear Arms

Lgs and Pygopus have so far mainly been analyzed in *Drosophila.* While those studies clearly show that both proteins are very important for Wg signaling, little is known so far about their significance in the vertebrate pathway. It was shown in zebrafish that B9L (BCL9-like or BCL9.2) is required for the induction of the T-box transcription factor tbx6, a Wnt8 target gene [55]. It will be very interesting to see what the phenotypes of mouse *lgs* and *pygo* knock-outs are. Will the NTAA of β-catenin play an equally essential role for Wnt pathway output as the CTAA? Or are there species-specific differences regarding their requirements? For the CTAA, future studies will shed more light on the C-terminal interactors. Is their order of action constant and critical, or can they transcriptionally prepare and activate Wnt targets by variable means? What about genes repressed by Wnt signaling? Is Arm/β-catenin also able to function as a Wnt signal-dependent repressor of gene transcription, or are Wnt-repressed genes only indirectly regulated by nuclear Arm/β-catenin? Clearly, resolving the unclear

issues of nuclear Arms will necessitate efforts in different fields. The reward may not only be to understand, but also to interfere with, aberrant pathway activity.

References

1. Radtke, F., and Clevers, H. (2005). Self-renewal and cancer of the gut: two sides of a coin. Science *307*, 1904-1909.
2. Reya, T., and Clevers, H. (2005). Wnt signalling in stem cells and cancer. Nature *434*, 843-850.
3. Moon, R.T., Kohn, A.D., De Ferrari, G.V., and Kaykas, A. (2004). WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet *5*, 691-701.
4. Logan, C.Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol *20*, 781-810.
5. Gates, J., and Peifer, M. (2005). Can 1000 reviews be wrong? Actin, alpha-Catenin, and adherens junctions. Cell *123*, 769-772.
6. Huber, A.H., Nelson, W.J., and Weis, W.I. (1997). Three-dimensional structure of the armadillo repeat region of beta-catenin. Cell *90*, 871-882.
7. Orsulic, S., and Peifer, M. (1996). An in vivo structure-function study of armadillo, the beta-catenin homologue, reveals both separate and overlapping regions of the protein required for cell adhesion and for wingless signaling. J Cell Biol *134*, 1283-1300.
8. Hoffmans, R., and Basler, K. (2004). Identification and in vivo role of the Armadillo-Legless interaction. Development *131*, 4393-4400.
9. Korswagen, H.C., Herman, M.A., and Clevers, H.C. (2000). Distinct beta-catenins mediate adhesion and signalling functions in C. elegans. Nature *406*, 527-532.
10. Polakis, P. (2002). Casein kinase 1: a Wnt'er of disconnect. Curr Biol *12*, R499-R501.
11. Behrens, J., von Kries, J.P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. Nature *382*, 638-642.
12. Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. Cell *86*, 391-399.
13. Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B.G., and Kemler, R. (1996). Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. Mech Dev *59*, 3-10.
14. Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S.C., Grosschedl, R., and Bienz, M. (1997). LEF-1, a nuclear factor coordinating signaling inputs from wingless and decapentaplegic. Cell *88*, 777-787.
15. van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., and Clevers, H. (1997). Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. Cell *88*, 789-799.
16. Brunner, E., Peter, O., Schweizer, L., and Basler, K. (1997). pangolin encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in Drosophila. Nature *385*, 829-833.
17. Cox, R.T., Pai, L.M., Miller, J.R., Orsulic, S., Stein, J., McCormick, C.A., Audeh, Y., Wang, W., Moon, R.T., and Peifer, M. (1999). Membrane-tethered Drosophila Armadillo cannot transduce Wingless signal on its own. Development *126*, 1327-1335.
18. Hsu, S.C., Galceran, J., and Grosschedl, R. (1998). Modulation of transcriptional regulation by LEF-1 in response to Wnt-1 signaling and association with beta-catenin. Mol Cell Biol *18*, 4807-4818.
19. Miller, J.R., and Moon, R.T. (1997). Analysis of the signaling activities of localization mutants of beta-catenin during axis specification in Xenopus. J Cell Biol *139*, 229-243.
20. Townsley, F.M., Cliffe, A., and Bienz, M. (2004). Pygopus and Legless target Armadillo/beta-catenin to the nucleus to enable its transcriptional co-activator function. Nat Cell Biol *6*, 626-633.

21. Yokoya, F., Imamoto, N., Tachibana, T., and Yoneda, Y. (1999). beta-catenin can be transported into the nucleus in a Ran-unassisted manner. Mol Biol Cell *10*, 1119-1131.
22. Fagotto, F., Gluck, U., and Gumbiner, B.M. (1998). Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of beta-catenin. Curr Biol *8*, 181-190.
23. Tolwinski, N.S., and Wieschaus, E. (2001). Armadillo nuclear import is regulated by cytoplasmic anchor Axin and nuclear anchor dTCF/Pan. Development *128*, 2107-2117.
24. Parker, D.S., Jemison, J., and Cadigan, K.M. (2002). Pygopus, a nuclear PHD-finger protein required for Wingless signaling in Drosophila. Development *129*, 2565-2576.
25. Belenkaya, T.Y., Han, C., Standley, H.J., Lin, X., Houston, D.W., and Heasman, J. (2002). pygopus Encodes a nuclear protein essential for wingless/Wnt signaling. Development *129*, 4089-4101.
26. Koike, M., Kose, S., Furuta, M., Taniguchi, N., Yokoya, F., Yoneda, Y., and Imamoto, N. (2004). beta-Catenin shows an overlapping sequence requirement but distinct molecular interactions for its bidirectional passage through nuclear pores. J Biol Chem *279*, 34038-34047.
27. Wiechens, N., and Fagotto, F. (2001). CRM1- and Ran-independent nuclear export of beta-catenin. Curr Biol *11*, 18-27.
28. Cong, F., and Varmus, H. (2004). Nuclear-cytoplasmic shuttling of Axin regulates subcellular localization of beta-catenin. Proc Natl Acad Sci U S A *101*, 2882-2887.
29. Rosin-Arbesfeld, R., Townsley, F., and Bienz, M. (2000). The APC tumour suppressor has a nuclear export function. Nature *406*, 1009-1012.
30. Neufeld, K.L., Zhang, F., Cullen, B.R., and White, R.L. (2000). APC-mediated downregulation of beta-catenin activity involves nuclear sequestration and nuclear export. EMBO Rep *1*, 519-523.
31. Henderson, B.R. (2000). Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. Nat Cell Biol *2*, 653-660.
32. Krieghoff, E., Behrens, J., and Mayr, B. (2006). Nucleo-cytoplasmic distribution of {beta}-catenin is regulated by retention. J Cell Sci *119*, 1453-1463.
33. Taipale, J., and Beachy, P.A. (2001). The Hedgehog and Wnt signalling pathways in cancer. Nature *411*, 349-354.
34. Booth, C., and Potten, C.S. (2000). Gut instincts: thoughts on intestinal epithelial stem cells. J Clin Invest *105*, 1493-1499.
35. Bienz, M., and Clevers, H. (2000). Linking colorectal cancer to Wnt signaling. Cell *103*, 311-320.
36. Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S., and Hedge, P. (1991). Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science *253*, 665-669.
37. Kinzler, K.W., Nilbert, M.C., Su, L.K., Vogelstein, B., Bryan, T.M., Levy, D.B., Smith, K.J., Preisinger, A.C., Hedge, P., McKechnie, D., and et al. (1991). Identification of FAP locus genes from chromosome 5q21. Science *253*, 661-665.
38. He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B., and Kinzler, K.W. (1998). Identification of c-MYC as a target of the APC pathway. Science *281*, 1509-1512.
39. Watson, S.A., and Smith, A.M. (2001). Hypergastrinemia promotes adenoma progression in the APC(Min-/+) mouse model of familial adenomatous polyposis. Cancer Res *61*, 625-631.
40. Zhang, T., Otevrel, T., Gao, Z., Ehrlich, S.M., Fields, J.Z., and Boman, B.M. (2001). Evidence that APC regulates survivin expression: a possible mechanism contributing to the stem cell origin of colon cancer. Cancer Res *61*, 8664-8667.
41. Hlubek, F., Jung, A., Kotzor, N., Kirchner, T., and Brabletz, T. (2001). Expression of the invasion factor laminin gamma2 in colorectal carcinomas is regulated by beta-catenin. Cancer Res *61*, 8089-8093.
42. Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O., and Clevers, H. (1998). The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. Nature *395*, 608-612.

43. Cavallo, R.A., Cox, R.T., Moline, M.M., Roose, J., Polevoy, G.A., Clevers, H., Peifer, M., and Bejsovec, A. (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. Nature *395*, 604-608.
44. Valenta, T., Lukas, J., and Korinek, V. (2003). HMG box transcription factor TCF-4's interaction with CtBP1 controls the expression of the Wnt target Axin2/Conductin in human embryonic kidney cells. Nucleic Acids Res *31*, 2369-2380.
45. Cuilliere-Dartigues, P., El-Bchiri, J., Krimi, A., Buhard, O., Fontanges, P., Flejou, J.F., Hamelin, R., and Duval, A. (2006). TCF-4 isoforms absent in TCF-4 mutated MSI-H colorectal cancer cells colocalize with nuclear CtBP and repress TCF-4-mediated transcription. Oncogene.
46. Brannon, M., Brown, J.D., Bates, R., Kimelman, D., and Moon, R.T. (1999). XCtBP is a XTcf-3 co-repressor with roles throughout Xenopus development. Development *126*, 3159-3170.
47. Hamada, F., and Bienz, M. (2004). The APC tumor suppressor binds to C-terminal binding protein to divert nuclear beta-catenin from TCF. Dev Cell *7*, 677-685.
48. Chen, G., Fernandez, J., Mische, S., and Courey, A.J. (1999). A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development. Genes Dev *13*, 2218-2230.
49. Schweizer, L., Nellen, D., and Basler, K. (2003). Requirement for Pangolin/dTCF in Drosophila Wingless signaling. Proc Natl Acad Sci U S A *100*, 5846-5851.
50. Daniels, D.L., and Weis, W.I. (2005). Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. Nat Struct Mol Biol *12*, 364-371.
51. Chinnadurai, G. (2002). CtBP, an unconventional transcriptional corepressor in development and oncogenesis. Mol Cell *9*, 213-224.
52. Graham, T.A., Weaver, C., Mao, F., Kimelman, D., and Xu, W. (2000). Crystal structure of a beta-catenin/Tcf complex. Cell *103*, 885-896.
53. Kramps, T., Peter, O., Brunner, E., Nellen, D., Froesch, B., Chatterjee, S., Murone, M., Zullig, S., and Basler, K. (2002). Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. Cell *109*, 47-60.
54. Städeli, R., and Basler, K. (2005). Dissecting nuclear Wingless signalling: recruitment of the transcriptional co-activator Pygopus by a chain of adaptor proteins. Mech Dev *122*, 1171-1182.
55. Brembeck, F.H., Schwarz-Romond, T., Bakkers, J., Wilhelm, S., Hammerschmidt, M., and Birchmeier, W. (2004). Essential role of BCL9-2 in the switch between beta-catenin's adhesive and transcriptional functions. Genes Dev *18*, 2225-2230.
56. Barker, N., Hurlstone, A., Musisi, H., Miles, A., Bienz, M., and Clevers, H. (2001). The chromatin remodelling factor Brg-1 interacts with beta-catenin to promote target gene activation. Embo J *20*, 4935-4943.
57. Hecht, A., Litterst, C.M., Huber, O., and Kemler, R. (1999). Functional characterization of multiple transactivating elements in beta-catenin, some of which interact with the TATA-binding protein in vitro. J Biol Chem *274*, 18017-18025.
58. Hecht, A., Vleminckx, K., Stemmler, M.P., van Roy, F., and Kemler, R. (2000). The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. Embo J *19*, 1839-1850.
59. Takemaru, K.I., and Moon, R.T. (2000). The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression. J Cell Biol *149*, 249-254.
60. Mosimann, C., Hausmann, G., and Basler, K. (2006). Parafibromin/Hyrax activates Wnt/Wg target genes by direct association with beta-catenin/Armadillo. Cell, in press.
61. Kim, S., Xu, X., Hecht, A., and Boyer, T.G. (2006). Mediator is a transducer of Wnt/beta -catenin signaling. J Biol Chem., in press.
62. Thompson, B.J. (2004). A complex of Armadillo, Legless, and Pygopus coactivates dTCF to activate wingless target genes. Curr Biol *14*, 458-466.
63. Cox, R.T., Pai, L.M., Kirkpatrick, C., Stein, J., and Peifer, M. (1999). Roles of the C terminus of Armadillo in Wingless signaling in Drosophila. Genetics *153*, 319-332.
64. Ma, H., Nguyen, C., Lee, K.S., and Kahn, M. (2005). Differential roles for the coactivators CBP and p300 on TCF/beta-catenin-mediated survivin gene expression. Oncogene *24*, 3619-3631.

65. Sims, R.J., 3rd, Belotserkovskaya, R., and Reinberg, D. (2004). Elongation by RNA polymerase II: the short and long of it. Genes Dev *18*, 2437-2468.
66. Kim, Y.J., Bjorklund, S., Li, Y., Sayre, M.H., and Kornberg, R.D. (1994). A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. Cell *77*, 599-608.
67. Kelleher, R.J., 3rd, Flanagan, P.M., and Kornberg, R.D. (1990). A novel mediator between activator proteins and the RNA polymerase II transcription apparatus. Cell *61*, 1209-1215.
68. Flanagan, P.M., Kelleher, R.J., 3rd, Sayre, M.H., Tschochner, H., and Kornberg, R.D. (1991). A mediator required for activation of RNA polymerase II transcription in vitro. Nature *350*, 436-438.
69. Malik, S., and Roeder, R.G. (2005). Dynamic regulation of pol II transcription by the mammalian Mediator complex. Trends Biochem Sci *30*, 256-263.
70. Conaway, J.W., Florens, L., Sato, S., Tomomori-Sato, C., Parmely, T.J., Yao, T., Swanson, S.K., Banks, C.A., Washburn, M.P., and Conaway, R.C. (2005). The mammalian Mediator complex. FEBS Lett *579*, 904-908.
71. Conaway, R.C., Sato, S., Tomomori-Sato, C., Yao, T., and Conaway, J.W. (2005). The mammalian Mediator complex and its role in transcriptional regulation. Trends Biochem Sci *30*, 250-255.
72. Sierra, J., Yoshida, T., Joazeiro, C.A., and Jones, K.A. (2006). The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes. Genes Dev *20*, 586-600.
73. Bauer, A., Huber, O., and Kemler, R. (1998). Pontin52, an interaction partner of beta-catenin, binds to the TATA box binding protein. Proc Natl Acad Sci U S A *95*, 14787-14792.
74. Bauer, A., Chauvet, S., Huber, O., Usseglio, F., Rothbacher, U., Aragnol, D., Kemler, R., and Pradel, J. (2000). Pontin52 and reptin52 function as antagonistic regulators of beta-catenin signalling activity. Embo J *19*, 6121-6130.
75. Rottbauer, W., Saurin, A.J., Lickert, H., Shen, X., Burns, C.G., Wo, Z.G., Kemler, R., Kingston, R., Wu, C., and Fishman, M. (2002). Reptin and pontin antagonistically regulate heart growth in zebrafish embryos. Cell *111*, 661-672.
76. Feng, Y., Lee, N., and Fearon, E.R. (2003). TIP49 regulates beta-catenin-mediated neoplastic transformation and T-cell factor target gene induction via effects on chromatin remodeling. Cancer Res *63*, 8726-8734.
77. Thompson, B., Townsley, F., Rosin-Arbesfeld, R., Musisi, H., and Bienz, M. (2002). A new nuclear component of the Wnt signalling pathway. Nat Cell Biol *4*, 367-373.
78. Tolwinski, N.S., and Wieschaus, E. (2004). A nuclear escort for beta-catenin. Nat Cell Biol *6*, 579-580.
79. Hoffmans, R., Stadeli, R., and Basler, K. (2005). Pygopus and legless provide essential transcriptional coactivator functions to armadillo/beta-catenin. Curr Biol *15*, 1207-1211.
80. Narlikar, G.J., Fan, H.Y., and Kingston, R.E. (2002). Cooperation between complexes that regulate chromatin structure and transcription. Cell *108*, 475-487.
81. Tutter, A.V., Fryer, C.J., and Jones, K.A. (2001). Chromatin-specific regulation of LEF-1-beta-catenin transcription activation and inhibition in vitro. Genes Dev *15*, 3342-3354.
82. Myers, L.C., and Kornberg, R.D. (2000). Mediator of transcriptional regulation. Annu Rev Biochem *69*, 729-749.
83. Malik, S., and Roeder, R.G. (2000). Transcriptional regulation through Mediator-like coactivators in yeast and metazoan cells. Trends Biochem Sci *25*, 277-283.
84. Rozenblatt-Rosen, O., Hughes, C.M., Nannepaga, S.J., Shanmugam, K.S., Copeland, T.D., Guszczynski, T., Resau, J.H., and Meyerson, M. (2005). The parafibromin tumor suppressor protein is part of a human Paf1 complex. Mol Cell Biol *25*, 612-620.
85. Yart, A., Gstaiger, M., Wirbelauer, C., Pecnik, M., Anastasiou, D., Hess, D., and Krek, W. (2005). The HRPT2 tumor suppressor gene product parafibromin associates with human PAF1 and RNA polymerase II. Mol Cell Biol *25*, 5052-5060.
86. Krogan, N.J., Dover, J., Wood, A., Schneider, J., Heidt, J., Boateng, M.A., Dean, K., Ryan, O.W., Golshani, A., Johnston, M., Greenblatt, J.F., and Shilatifard, A. (2003).

The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. Mol Cell *11*, 721-729.

87. Shi, Y., Sawada, J., Sui, G., Affar el, B., Whetstine, J.R., Lan, F., Ogawa, H., Luke, M.P., Nakatani, Y., and Shi, Y. (2003). Coordinated histone modifications mediated by a CtBP co-repressor complex. Nature *422*, 735-738.
88. Bienz, M. (2002). The subcellular destinations of APC proteins. Nat Rev Mol Cell Biol *3*, 328-338.
89. Henderson, B.R., and Fagotto, F. (2002). The ins and outs of APC and beta-catenin nuclear transport. EMBO Rep *3*, 834-839.
90. van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A.P., Tjon-Pon-Fong, M., Moerer, P., van den Born, M., Soete, G., Pals, S., Eilers, M., Medema, R., and Clevers, H. (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell *111*, 241-250.

2. Results & Discussion

2.1 Identification and in vivo role of the Armadillo-legless interaction

Raymond Hoffmans and Konrad Basler (2004)
Development. 131, 4393-4400.

Research article Development and disease 4393

Identification and in vivo role of the Armadillo-Legless interaction

Raymond Hoffmans and Konrad Basler*

Institut für Molekularbiologie, Universität Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland
*Author for correspondence (e-mail: basler@molbio.unizh.ch)

Accepted 8 June 2004

Development 131, 4393-4400
Published by The Company of Biologists 2004
doi:10.1242/dev.01296

Summary

The Wnt signalling system controls many fundamental processes during animal development and its deregulation has been causally linked to colorectal cancer. Transduction of Wnt signals entails the association of β-catenin with nuclear TCF DNA-binding factors and the subsequent activation of target genes. Using genetic assays in *Drosophila*, we have recently identified a presumptive adaptor protein, Legless (Lgs), that binds to β-catenin and mediates signalling activity by recruiting the transcriptional activator Pygopus (Pygo). Here, we characterize the β-catenin/Lgs interaction and show: (1) that it is critically dependent on two acidic amino acid residues in the first Armadillo repeat of β-catenin; (2) that it is spatially and functionally separable from the binding sites for TCF factors, APC and E-cadherin; (3) that it is required in endogenous as well as constitutively active forms of β-catenin for Wingless signalling output in *Drosophila*; and (4) that in its absence animals develop with the same phenotypic consequences as animals lacking Lgs altogether. Based on these findings, and because Lgs and Pygo have human homologues that can substitute for their *Drosophila* counterparts, we infer that the β-catenin/Lgs binding site may thus serve as an attractive drug target for therapeutic intervention in β-catenin-dependent cancer progression.

Key words: *Drosophila*, Disease, Colorectal cancer, Wnt signalling, β-catenin

Introduction

Colorectal cancer is the second leading cause of cancer incidence and cancer death among adults in Europe. It is estimated for the year 2000 alone that 362,620 people were diagnosed with colorectal cancer, and 198,778 patients died owing to this disease (Ferlay et al., 2001). In more than 80% of the sporadic colorectal cancers, both alleles of the Adenomatous Polyposis Coli (*APC*) gene are inactivated (Kinzler and Vogelstein, 1996). The APC protein forms – together with Axin and GSK3β – a degradation complex for β-catenin. In this complex, GSK3β phosphorylates β-catenin, which in turn is ubiquitinated and thereby targeted for destruction. Wnt signalling inhibits the degradation complex and hence leads to the cytoplasmic accumulation and entry of β-catenin into the nucleus, where it forms a complex with members of the Pangolin(Pan)/TCF/Lef family of DNA-binding proteins, the putative adaptor protein Legless/BCL9 (Lgs) and the transcriptional regulator Pygopus (Pygo) (Behrens et al., 1996; Belenkaya et al., 2002; Brunner et al., 1997; Kramps et al., 2002; Parker et al., 2002; Riese et al., 1997; Thompson et al., 2002; van de Wetering et al., 1997). Loss of APC also causes an increase in β-catenin levels and thus leads to a constitutive activation of this pathway. The nuclear β-catenin complex activates transcription of known proto-oncogenes such as *Myc* and *cyclin D1* (He et al., 1998; Shtutman et al., 1999). Preventing the formation of the TCF/β-catenin/Lgs/Pygo complex should halt the expression of these genes and thus alleviate the detrimental effects caused by the loss of the APC tumour suppressor. Indeed, overexpression of a dominant-negative form of TCF4 in colorectal cancer cells results in G1 cell cycle arrest (van de Wetering et al., 2002). Furthermore, reduction of human Pygo expression by means of RNA interference in colorectal cancer cells reduces the transcriptional output induced by the nuclear β-catenin complex (Thompson et al., 2002).

Here, we set out to characterize the interaction of β-catenin and Lgs. We report the identification of two amino acids of β-catenin that play an essential role in Lgs binding. This presumed binding site is specific for Lgs and is not required for APC, E-cadherin or TCF4. We show that Armadillo (Arm), the *Drosophila* homologue of β-catenin, depends on these amino acid residues for mediating Wnt/Wg signalling in vivo, but not for establishing functional adherens junctions. Together, our results indicate that the β-catenin/Lgs interaction may provide an attractive target for therapeutic intervention.

Materials and methods

β-catenin and Arm mutants

The crystal structure of β-catenin (Protein Data Bank accession code 2BCT) was used in conjunction with the PDB viewer (http://au.expasy.org/spdbv) to select amino acid residues of β-catenin that are exposed and have more than 30% accessibility. Twenty-five human β-catenin mutants (in pGAD424) were obtained from von Kries et al. (von Kries et al., 2000). These constructs comprised only Arm repeats 3-12 and the C terminus, and were extended with Arm repeat 1 and 2, such that all final constructs extended from amino acids 129 to 781. Another 14-point mutations were introduced in repeats 1-3 by site-directed mutagenesis (Quickchange-Kit, Stratagene). Human LGS1 (amino acids 199-392), human TCF4 (amino acids 1-130), mouse Apc (amino acids 1152-1393), mouse E-cadherin (amino acids 773-885) and mouse α-catenin (amino acids 1-750) were cloned into pBTM116 (Bartel and Fields, 1997). For interactions with α-catenin the mutations were brought into the context of full length β-catenin constructs. All constructs were verified by sequencing.

A subset of the β-catenin mutations were also introduced into Arm.

For simplicity, we use the amino acid numbering of β-catenin for both β-catenin and Arm throughout the text. β-Catenin D162, E163, D164 and K435 would correspond to Arm D170, E171, D172 and K443.

Yeast two-hybrid assays

The yeast two-hybrid system as described previously (Bartel and Fields, 1997) was used. Interactions between proteins were measured using the quantitative 'Liquid Culture Assay Using ONPG as Substrate' (Clontech, 2001).

Transgenes

For embryonic experiments *arm* transgenes were expressed from UAS-constructs under control of the *daughterless-Gal4* driver (Wodarz et al., 1995). Three independent lines were established and tested for the arm^{S10}-*wt*, arm^{S10}-*D164A* and arm^{S10}-*K435E* constructs, and two independent lines for *Δarm-wt*, *Δarm-D164A* and *Δarm-K435E*; in all cases, different integrations of the same construct yielded similar effects. For rescue experiments full-length *arm* transgenes were driven by the *tubulinα1* (*tub*) promoter (Basler and Struhl, 1994). All *arm*-coding regions used contain a *Myc* epitope in their C-terminal region at the same position as the arm^{S10} construct used by Pai et al. (Pai et al., 1997).

Germline clones

To obtain *arm* germline clones, second and third instar larvae generated from a cross between arm^{2a9} *FRT101*/*FM7*; *tub-arm[-wt or –D164A]*/+ virgins with ovo^{D1} *FRT101*/*Y*; *hs-flp[F38]*/*hs-flp[F38]* were heat-shocked at 38°C for 1.5 hours. After hatching, the fertile females will produce only progeny from arm^{2a9} mutant germlines. Females bearing the *tub-arm-wt* transgene were crossed to *y w* males and laid embryos that all contain maternal *tub-arm-wt* product (otherwise no eggs would be generated). Only 25% of these embryos will inherit neither the rescuing transgene nor the paternal arm^{+} allele, and these embryos resemble zygotic arm^{2a9} embryos. The observed number for such embryos was 90 out of 385. Females that were arm^{2a9}/arm^{2a9}; *tub-arm-D164A*/+ were crossed with *y w* males and laid embryos that all contain maternal *tub-arm-D164A* product (otherwise no eggs would be generated). Three classes of embryos are expected (50% class I, 25% class II, 25% class III): while all embryos are maternally mutant for arm^{2a9}, 50% of them (class I) are zygotically arm^{+}, and hence rescued (from the paternal X chromosome), and the other 50% (classes II and III) are also zygotically mutant for arm^{2a9}. Half of these (class II), however, inherit the *tub-arm-D164A* transgene, and show a slightly weaker segment polarity phenotype (Fig. 5C) compared with the other half (class III) that does not (Fig. 5D). The observed numbers for these three classes were 33, 12 and 14, respectively. For the generation of *lgs* germline clones see Kramps et al. (Kramps et al., 2002).

Disc clones

Mutant imaginal disc clones were generated by crossing arm^{2a9} *FRT18*/*FM7* females with *hs-flp hs-GFP FRT18*; *tub-arm[-wt or -D164A]*/TM6b. Ninety-six hours after egg laying, larvae were heat shocked at 38°C for 1 hour. Female larvae that did not carry *TM6b* were dissected 48 hours after the heat shock. Imaginal discs were fixed and stained by standard techniques. Antibodies used were mouse monoclonal anti-Dll (gift from I. Duncan), rat monoclonal anti-dE-cadherin (DCAD2, gift from T. Uemura) and rabbit polyclonal anti-Lgs (Kramps et al., 2002).

Results

Identification and characterization of the Lgs binding site of β-catenin

The primary structure of β-catenin consists of acidic N and C termini, and a highly basic central region containing 12 imperfect sequence repeats that are known as Armadillo repeats (Arm repeats). These repeats pack against each other to form a continuous superhelix, which features a positively charged groove (Huber et al., 1997). The Arm repeat domain provides binding sites for APC, Axin, E-cadherin, TCF4 and human LGS1 (Fig. 1A). Despite lack of significant sequence homology, APC, E-cadherin and TCF4 are known to bind competitively to β-catenin (Hulsken et al., 1994; Omer et al., 1999). Structural studies have shown that APC, E-cadherin and TCF4 bind to largely overlapping regions of the positively charged groove of β-catenin (Eklof Spink et al., 2001; Graham et al., 2000; Graham et al., 2001; Huber and Weis, 2001; Xing et al., 2003). Lgs instead requires the first four Arm repeats for binding to Arm (Kramps et al., 2002). We set out to map and characterize the human LGS1-β-catenin interaction by performing an alanine mutagenesis scan.

A set of 39 β-catenin mutants containing substitutions of single, exposed amino acid residues (mostly with basic and aromatic side chains, Fig. 1C,D) was tested for the ability to bind human LGS1 in a yeast two-hybrid system (see Materials and methods). Two of these mutants showed a reproducible reduction in binding: the D162A mutation reduced binding by fourfold compared with wild-type β-catenin and D164A even by 25-fold. In the initial screen, R386A also showed a reduced binding to human LGS1 (two-fold, Fig. 1E), but this reduction was variable (Fig. 1F) and is not considered to be significant.

In addition to its role in Wnt signalling, β-catenin is also a component of the cadherin-based cell adhesion system, linking the transmembrane protein E-cadherin to α-catenin, thereby connecting adherens junctions to the cytoskeleton (reviewed by Pokutta and Weis, 2002). β-Catenin is also part of its own degradation complex consisting of APC, Axin and GSK3β. In order to evaluate the specificity of the mutations that disrupt β-catenin/human LGS1 binding, mutations D162A and D164A were tested for their effect on the interactions between β-catenin and APC, E-cadherin, TCF4 and α-catenin. As a control, we also included in this analysis E163A, which had virtually no effect on the β-catenin/human LGS1 interaction even though it also reduces the negative charge at this region of the protein. None of the three mutations affected the binding of β-catenin to APC, E-cadherin or TCF4. Binding to α-catenin, however, was reduced approximately twofold by D162A and D164A (Fig. 1F). This was unexpected, as the region comprising amino acids 120-151 of β-catenin has been shown to be necessary and sufficient for binding to α-catenin (Aberle et al., 1994). Amino acids D162, E163 and D164 form an acidic knob in repeat 1 of β-catenin (Fig. 1B, white arrow), on the side opposite to the basic groove. From these results we conclude: (1) that β-catenin binds human LGS1 and APC/E-cadherin/TCF4 on opposite sides; and (2) that the binding to human LGS1 but not to APC/E-cadherin/TCF4 is disrupted by the mutations D162A and D164A.

Arm-D164A fails to rescue *armadillo* null mutant animals

Armadillo (Arm) is the *Drosophila* homologue of β-catenin. The two proteins show high sequence similarity, especially in the Arm repeat region (Peifer and Wieschaus, 1990; Peifer et al., 1994). To investigate whether a mutant form of Arm, which can no longer bind Lgs, has impaired transcriptional activity in *Drosophila*, we first analyzed whether β-catenin and Arm use

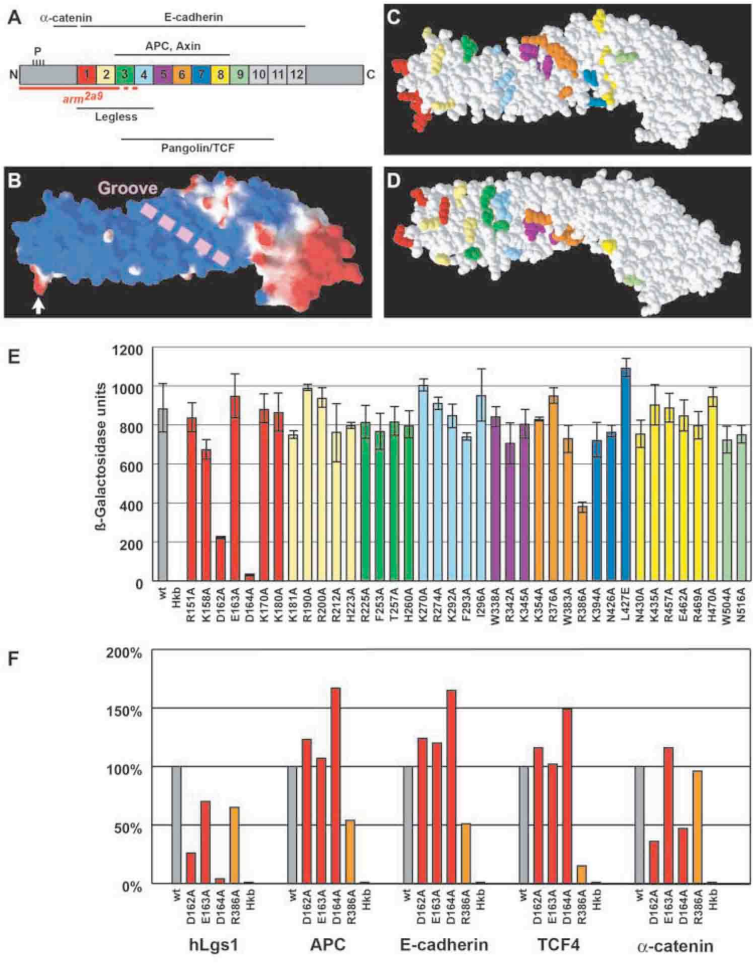

Fig. 1. Identification of mutations in β-catenin that affect Lgs binding. (A) Schematic representation of the β-catenin protein. The Arm repeats are marked by different colours and numbered 1-12. Black lines represent the binding domains of β-catenin interaction partners. P marks the phosphorylation sites used by the degradation complex. The red line indicates the protein product of the arm^{2a9} allele, which contains an X-ray induced frame shift in Arm repeat 3 and results at best in a truncated protein. (B) Electrostatic surface of β-catenin. Blue and red surfaces represent regions of positive (basic) and negative (acidic) potential, respectively. White arrow indicates the acidic knob that is essential for Lgs binding (amino acids 162 to 164). The broken line indicates the basic groove in which E-cadherin, TCF4 and APC make multiple contacts with β-catenin (reviewed by Daniels et al., 2001). (C,D) Space filling models of Arm repeats 1-12. The mutations are indicated in the colour of the Arm repeat that contains the mutation (same colour scheme as in A). The model in D is turned by 90° along the horizontal axis compared with that in C. (E) Interaction of mutant β-catenin proteins with human LGS1 tested by yeast two-hybrid analysis. Mutations D162A, D164A and R386A show an effect on Lgs binding. Bars are colour-coded to match the colour scheme of the Arm repeats in A. The protein Huckebein (Hkb) served as a negative control, as it is a transcription factor (Bronner et al., 1994) that plays no role in Wnt/Wg signalling. (F) A subset of the β-catenin mutants was tested for binding to APC, E-cadherin, TCF4 and α-catenin. D162A and D164A do not have a negative effect on binding of β-catenin to APC, E-cadherin and TCF4. The mutations that affected Lgs binding also reduced α-catenin binding by 50%. R386A affected Lgs binding to variable degree (compare E with F), but led to a reproducible reduction in the binding to APC, E-cadherin and TCF. Results are presented as the percentage of binding compared with wild-type β-catenin.

equivalent sites for binding human and *Drosophila* Lgs. The D164A mutation – and as a negative control the E163A mutation – were introduced into Arm and found to affect the Arm/Lgs interaction to the same extent as the corresponding mutations in β-catenin (not shown). We then tested whether Arm-D164A can substitute for the wild-type form of Arm in vivo by performing a rescue assay with the arm^{2a9} allele, which has a frameshift mutation in Arm repeat 3 (Fig. 1A) and is the strongest *arm* allele known. Hemizygous arm^{2a9} males die as embryos but can be rescued by *tubulinα1*-promoter-driven *arm-wt* or *arm-E163A* transgenes to adulthood with no obvious phenotypes (Table 1). By contrast, arm^{2a9} males die as embryos or early larvae when these transgenes contain the D164A mutation or K435E, which affects TCF/Pan binding (Graham et al., 2000). We interpret these results to indicate that the wild-type function of Arm depends crucially on its ability to bind Lgs and Pan.

Constitutive signalling activity of Arm^{S10} depends on D164

Mutations in the N terminus of β-catenin that impede its phosphorylation and subsequent degradation cause – like loss of APC or Axin – constitutive activation of the Wnt/Wg pathway and are found in 10% of sporadic colorectal cancers (Sparks et al., 1998; Korinek et al., 1997; Morin et al., 1997). In *Drosophila*, embryonic overexpression of N-terminally truncated forms of Arm mimics this situation and leads to a naked cuticle phenotype, owing to overactivation of the pathway. Arm^{S10} is one such form, owing to an in-frame deletion that removes the GSK3β phosphorylation sites but

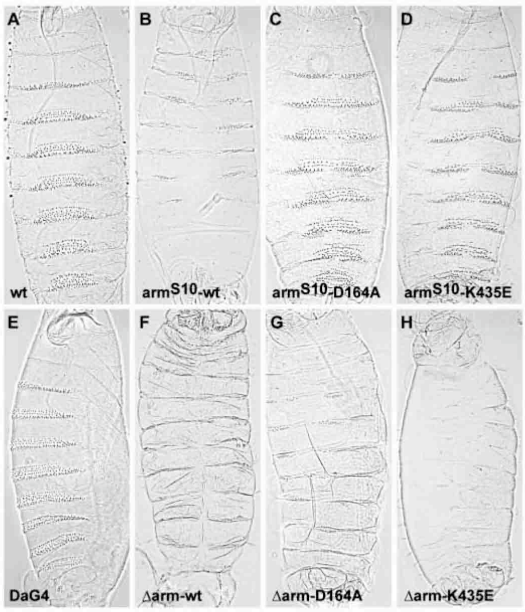

Fig. 2. Constitutively active forms of Arm depend on Lgs binding for signalling activity. (A) The cuticle of a wild-type (wt) embryo. (B) Ubiquitous expression of a constitutively active form of Arm (Arm^{S10}-wt) results in a naked cuticle phenotype. (C,D) Ubiquitous expression of Arm^{S10} carrying the D164A or the K435E mutations to impair the binding to Lgs or Pan, respectively, no longer causes a naked cuticle phenotype. Occasional ectopic denticles in areas where Wg is active (and which are normally naked) can be observed and indicate that these two mutant forms may exhibit slight dominant-negative activities, possibly by titrating away Pan and Lgs, respectively, from wild-type Arm. (E) The cuticle of an embryo containing the *daughterless-Gal4* (DaG4) driver is indistinguishable from that of wild-type embryos (A). (F-H) Ubiquitous expression of a constitutively active, membrane-targeted form of Arm (ΔArm-wt) results in a naked cuticle. Mutations in ΔArm that affect binding to Lgs (ΔArm-D164A) or Pan (ΔArm-K435E) still result in a naked cuticle phenotype, most probably because in this situation endogenous Arm, and not membrane-targeted Arm, mediates the signalling output (Tolwinski and Wieschaus, 2001; Tolwinski and Wieschaus, 2004). All transgenes in these experiments were controlled by UAS-promoters driven by DaG4 (Wodarz et al., 1995).

leaves α-catenin binding intact (Pai et al., 1997). Ubiquitous expression of Arm^{S10} (from a transgene driven by *daughterless-Gal4*) results in naked cuticle (Fig. 2B). However, overexpression of Arm^{S10}-D164A results in an almost wild-type cuticle (Fig. 2C). We interpret the correlation between the failure to suppress denticle formation and the failure to bind Lgs to indicate that Arm^{S10} largely depends on Lgs binding for its biological activity. As a control, we also overexpressed a form of Arm^{S10} that is affected in Pan binding (Arm^{S10}-K435E); the K435E mutation also efficiently reverts the gain-of-function activity of Arm^{S10} (Fig. 2D). Thus, the constitutive activity of Arm^{S10} depends on the binding of both Lgs and Pan.

Another stable and thus constitutively active form of Arm is ΔArm, in which a large N-terminal region comprising the GSK3β phosphorylation and α-catenin-binding sites is replaced by a myristoylation signal (Zecca et al., 1996). Its biological activity depends on the presence of wild-type cellular Arm (Tolwinski and Wieschaus, 2001; Tolwinski and Wieschaus, 2004). It has been shown that expression of membrane-tethered forms of β-catenin leads to the nuclear localization of endogenous β-catenin (Miller and Moon, 1997). If ΔArm signalling is mediated by wild-type cellular Arm, then disrupting the binding to Lgs or Pan should not affect its ability to activate the Wg pathway. Indeed, we find that overexpression of ΔArm-wt, ΔArm-D164A and ΔArm-K435E all resulted in a completely naked cuticle phenotype (Fig. 2F-H), suggesting that ΔArm signals via cellular Arm, which is wild-type and hence able to recruit Lgs.

Reduced expression levels of Wg targets in *arm-D164A* cells

To assess the role of the D164 site in the transcriptional function of Arm, we analyzed Wg target gene expression in *arm* mutant clones in third instar wing imaginal discs. There Wg is expressed at the dorsoventral boundary in a narrow stripe of cells and regulates the expression of a number of genes, among them *Distalless* (Dll), which is expressed in a broad band of cells on both sides of the wing margin (Diaz-Benjumea and Cohen, 1995; Zecca et al., 1996). We used the strong *arm* allele arm^{2a9} to induce mutant clones in the second larval instar. Dll expression was lost in these clones 48 hours later (Fig. 3B). Ubiquitous expression of the *tubulinα1-arm-wt* transgene fully restored Dll expression in such clones (Fig. 3C). By contrast, arm^{2a9} clones showed severely reduced Dll expression when the *tubulinα1-arm-D164A* transgene was used (Fig. 3D). Thus, Arm-D164A is severely impaired in transducing the Wg signal, suggesting that Arm needs to bind Lgs to efficiently upregulate Dll expression in response to larval Wg.

To exclude the possibility that *arm* mutant cells unspecifically shut down gene expression, we analyzed the protein levels of Lgs in *arm* clones. Lgs is a nuclear protein and its levels are not regulated by Wg signalling (Kramps et al., 2002). As shown in Fig. 3A, arm^{2a9} clones express Lgs to the same extent as wild-type cells. This control experiment

Table 1. Rescue ability of the *arm* transgene

Transgene	Rescue (%)	*n*
None	0	150
tub-arm-wt	96	166
tub-arm-E163A	62	81
tub-arm-D164A	0	133
tub-arm-K435E	0	66

D164 is required for Arm function. Females heterozygous for arm^{2a9} were crossed with males containing different *tubulinα1*-promoter-driven rescue constructs. The percentages of rescued males containing both the arm^{2a9} allele and the *tubulinα1*-rescue constructs are shown. *n* indicates the male progeny that contain the *tubulinα1*-rescue construct but inherited the wild-type *arm* allele from the balancer chromosome instead of the arm^{2a9} allele and therefore corresponds to the expected number of arm^{2a9} males with the *tubulinα1*-rescue construct.

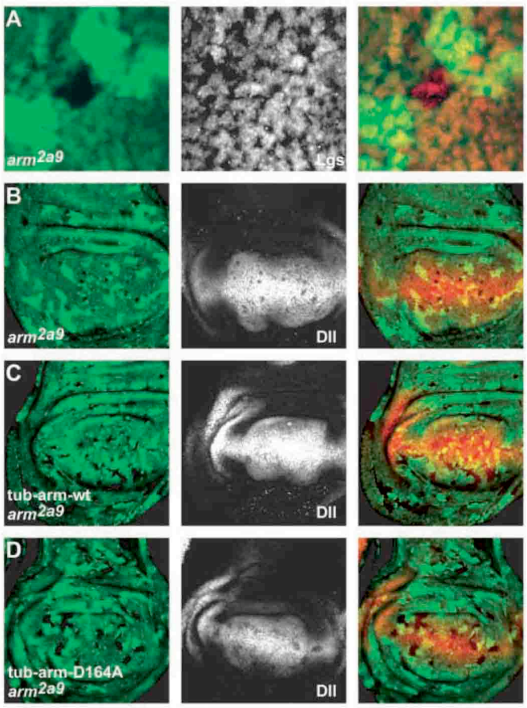

Fig. 3. Arm-D164A fails to restore wild-type levels of Dll expression in *arm*-null clones. Confocal sections of wing discs are shown, stained for either Lgs or Dll expression. Loss of GFP (green) marks clones lacking Arm (left panels). Merged images are shown towards the right. (A) arm^{2a9} clones express Lgs, indicating that these cells are still viable and capable of expressing proteins. Not only the overall levels, but also the subcellular distribution of Lgs is unaffected by the loss of Arm. The magnification in this panel is sixfold higher than those in B-D. (B) arm^{2a9} clones do not express Dll. (C) Ubiquitous expression of Arm-wt rescues Dll expression in arm^{2a9} clones. (D) Ubiquitous expression of Arm-D164A has only weak rescuing activity and most arm^{2a9} clones exhibit severely reduced levels of Dll expression.

indicates that the loss or reduction of Dll expression in *arm* null and *arm-D164A* clones is due to the inability of these cells to respond efficiently to Wg rather than due to a general effect caused by cellular decay. It further rules out the possibility that the nuclei of mutant cells have escaped the apicobasal plane at which target gene expression was optically recorded.

Arm-D164A can restore adherens junctions of *arm* mutant cells

We noticed that arm^{2a9} clones are not only smaller than their twin-spots but that their shape is round compared with the irregular outline of normal clones (Fig. 3B). On the contrary, *arm* clones that express *arm-wt* or *arm-D164A* transgenes are similar in size to their twin-spots and have an irregular shape (Fig. 3C,D). These differences hint at an adherens junction defect of *arm* null mutant cells, as β-catenin is required at these sites to link E-cadherin and α-catenin, and cells with defective adherens junctions or different E-cadherin levels sort out from neighbouring cells (Dahmann and Basler, 2000; Uemura et al., 1996). In order to visualize adherens junctions we stained wing discs with an antibody directed against *Drosophila* E-cadherin (Uemura et al., 1996). Indeed, E-cadherin distribution is diffuse in *arm* null clones (Fig. 4A). As *arm-wt* and *arm-D164A* transgenes rescue the abnormal distribution of dE-cadherin in *arm* cells (Fig. 4B,C), and no longer cause aggregation into non-intermingling cell groups, their products appear to restore the function of adherens junction. This suggests that Arm-D164A can confer β-catenin function at adherens junctions, and hence is able to sufficiently tether E-cadherin and α-catenin.

Replacement of maternal and zygotic Arm by Arm-D164A is equivalent to lack of Lgs function

Although both the failure to rescue zygotically mutant animals and the failure to rescue gene expression in *arm* mutant disc cells indicate a requirement for the D164 site and hence the Arm/Lgs interaction, neither of these assays provides the means to compare the reduction of Wg transduction to that caused by the genetic removal of Lgs. Additionally both assays may be influenced by the perdurance of wild-type *arm* product. The most stringent test for the role of D164 in Wg signalling is the creation of embryos in which both the maternal and the zygotic contribution of wild-type *arm* product are replaced by Arm-D164A. To achieve this situation, we generated germline clones using the arm^{2a9} allele. Such germline clones fail to produce eggs because of junctional defects (Peifer et al., 1993). However, arm^{2a9} germline clones do give rise to eggs and phenotypically normal larvae in the maternal and zygotic presence of a *tubulinα1-arm-wt* transgene (not shown). When only maternal *arm-wt* product is contributed, eggs are laid from mutant germline clones and the resulting embryos resemble zygotic arm^{2a9} embryos. Eggs were also laid from clones expressing the *tubulinα1-arm-D164A* transgene, corroborating our conclusion that Arm-D164A protein can restore functional adherens junctions (Fig. 5C,D). Embryos whose sole source of Arm, both maternally and zygotically, was the *tubulinα1-arm-D164A* transgene (Fig. 5C) died with a segment polarity phenotype characterized by an excess of ventral denticles at the expense of naked cuticle. This phenotype, which is weaker than that of *wg* null mutants (Nüsslein-Volhard et al., 1984; Bejsovec and Wieschaus, 1993), closely resembles the phenotype of embryos devoid of maternal and zygotic *lgs* function (Fig. 5B). Because the *arm-D164A* phenotype is not notably weaker than that of *lgs* embryos, we conclude that the D164A mutation effectively eliminates in vivo – as in the yeast assay – most or all Lgs function. Moreover, as we failed to observe any phenotypes of *arm-D164A* embryos that surpassed those of *lgs* embryos, we also conclude that the D164 site is unlikely to serve any critical function other than recruitment of Lgs.

Discussion

The Wnt signalling pathway not only controls a multitude of fundamental patterning processes during animal development (reviewed by Wodarz and Nusse, 1998), its deregulation is also

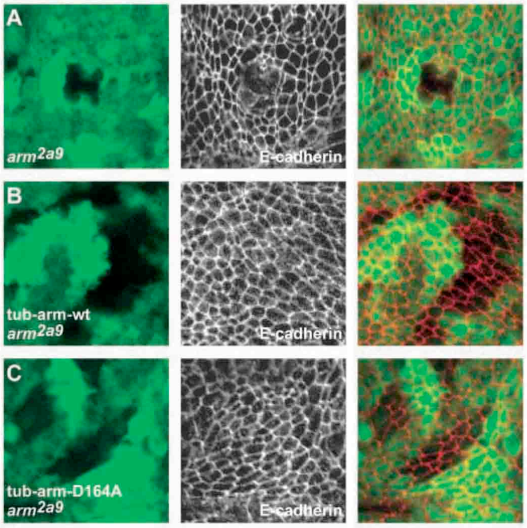

Fig. 4. Arm-D164A can restore functional adherens junctions in *arm* clones. Confocal sections of wing disc cells are shown, stained for E-cadherin expression (central panels) marking the adherens junctions. Loss of GFP (green) marks clones lacking Arm (left panels). Merged images are shown to the right. (A) arm^{2a9} clones are small, have an abnormally round shape, and show diffuse E-cadherin staining. (B) arm^{2a9} clones expressing Arm-wt exhibit the typical irregular outline of wild-type clones in wing discs and show restored E-cadherin staining. (C) arm^{2a9} clones expressing Arm-D164A behave like those expressing Arm-wt; they exhibit irregular outlines and restored E-cadherin staining.

responsible for an increasing number of cancers. Hence, major efforts strive towards the identification of all protein components involved in this pathway and also for the detailed characterization of their molecular interactions. We have recently identified two novel genes involved in the transduction of the Wg signal, *lgs* and *pygo*, and found that their products serve as adaptor proteins to convert nuclear β-catenin/Arm activity into transcriptional activation of target genes (Kramps et al., 2002). Here, we are concerned with the question of how β-catenin and Lgs interact molecularly with each other. Our analysis addressed three issues: localization of the binding site on β-catenin, specificity of this site vis-à-vis other partners of β-catenin and in vivo significance of this interaction for Wg signal transduction.

Binding site

By means of site-directed mutagenesis we assayed the role of conspicuous β-catenin residues in the binding to human LGS1. Two amino acids, D162 and D164, were identified that are both necessary for human LGS1 binding. Because substitutions of these residues with other amino acids did not affect the binding of several other proteins to β-catenin, we interpret their role as contact sites for human LGS1, rather than a structural function enhancing stability and/or three-dimensional conformation of β-catenin. This conclusion, however, will need to be confirmed by determining the crystal structure of the β-catenin/human LGS1 complex.

Specificity

We showed that neither D162 nor D164 is required for binding to APC, E-cadherin or TCF4. Substitutions of these amino acids did reduce binding to α-catenin twofold, but our in vivo data suggest that this reduction does not prevent the assembly of adherens junctions. The specificity of the β-catenin/human LGS1 interaction vis-à-vis that of β-catenin and APC, E-cadherin or TCF4 is consistent with their respective locations on the surface of β-catenin. While crystallographic studies showed that APC, E-cadherin and TCF4 all bind to a common, extended surface within the groove of β-catenin formed by Arm repeats 3-10 (reviewed by Daniels et al., 2001), our analysis indicates that human LGS1 binds an acidic knob in Arm repeat 1. This knob is not only located more N terminally, it is also situated on the side of β-catenin, which is opposite to the groove (Fig. 1B). The spatial separation of these binding sites is in agreement with their separable functions observed in our yeast binding assays, as well as with previous GST pull-down assays, in which we observed simultaneous binding of TCF4 and human LGS1 to β-catenin (Kramps et al., 2002).

In vivo significance

To assess the role of D162 and D164 in Wg transduction, we

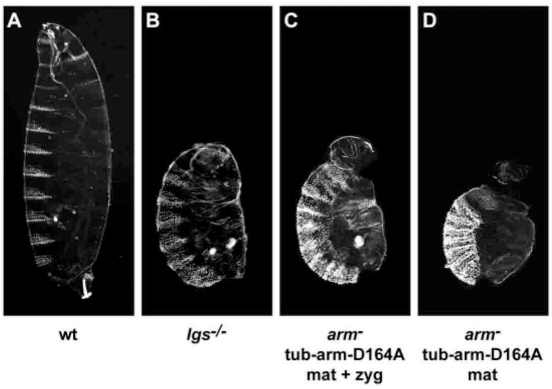

Fig. 5. *arm-D164A* animals closely resemble *lgs* mutants. (A) The cuticle of a wild-type (wt) embryo shown in dark-field. (B) Embryo maternally and zygotically mutant for lgs^{20F}. This allele carries a premature stop codon and is hence considered a null allele (Kramps et al., 2002). (C) Embryo representing class II (see Materials and methods), derived from an arm^{2a9} germline clone with maternal (mat) and zygotic (zyg) Arm-D164A function. Such embryos display a lawn of denticles similar to that of embryos that are maternally and zygotically mutant for the *lgs* null allele (B). (D) Embryo representing class III (see Materials and methods), derived from an arm^{2a9} germline clone with only maternal (mat) Arm-D164A function. Such embryos display a phenotype which is more severe than that of class II and *lgs* null embryos. Note that the reduced contrast in the denticle patterns of the embryos shown in A and B versus those in C and D stems from the marker gene *yellow*, which, for technical reasons (Kramps et al. 2002), is mutant in embryos A and B, but wild type in C and D.

subjected mutant forms of Arm to various assays designed to reveal their in vivo function. Simple rescue and overexpression experiments showed that transgenic Arm-D164A cannot substitute for endogenous Arm, and that the D164A mutation significantly reduces the constitutive signalling activity associated with N-terminal deletions of Arm. When tested in more advanced assays, we find that D164 is required by wing disc cells to maintain Wg target gene expression and by developing embryos for segmentation. Together, these experiments support the conclusion that Arm signalling function relies on its capability to bind to Lgs throughout development.

Although it is straightforward to interpret our results as a qualitative indication for the significance of the Arm/Lgs interaction, it is more difficult to assess their outcome in a quantitative manner. For example, the apparent residual expression of Dll in Arm-D164A cells may reflect perdurance of wild-type Arm or Dll proteins, but it could also indicate that a fraction of the Wg signal can be transmitted despite the D164A mutation. This latter scenario could in turn be attributed to some residual binding of Arm to Lgs, but it could also be explained by a partial redundancy of Lgs function. Lgs may be required for efficient Arm-mediated activation of Wg targets, but some activation may also occur in its absence. Consistent with this latter view, we have observed that animals lacking maternal and zygotic *lgs* product exhibit phenotypes equivalent to animals in which the sole source of Arm is the D164A transgene, yet neither of the two phenotypes are quite as severe as that of *wg*-null mutants.

Possible relevance for human cancer

The Wnt pathway is highly conserved between *Drosophila* and vertebrates. The human homologues of Lgs (LGS1/BCL9) and Pygo (PYGO1 and PYGO2) can rescue *lgs* and *pygo* mutant flies, respectively (Kramps et al., 2002). This suggests that these proteins have the same function in vertebrates and in *Drosophila*. It is possible therefore, that our in vivo data can be extrapolated to Wnt signalling in mammals.

Mutations in APC occur in more than 80% of inherited and sporadic colorectal cancers (Kinzler and Vogelstein, 1996). These mutations lead to accumulation of free β-catenin and as a result to overexpression of Wnt target genes. A chemical compound that interferes with the formation of the nuclear TCF/β-catenin/Lgs/Pygo complex should in theory halt the progression of cancer. Such an anti-cancer drug must be highly specific though, as it should only disrupt the nuclear β-catenin complex, but neither the cytoplasmic β-catenin/APC/Axin complex nor the β-catenin/E-cadherin complex at the cell membrane. APC, Axin and E-cadherin functions should not be compromised, as all three of them have tumour suppressor roles (reviewed by Giles et al., 2003). This is not the case, however, for TCF and Lgs. Crystal structure data indicates that APC, Axin, E-cadherin and TCF4 partly use of the same contact sites of β-catenin for their binding (Eklof Spink et al., 2001; Graham et al., 2000; Graham et al., 2001; Huber and Weis, 2001; Xing et al., 2003). Therefore, designing an inhibitor that specifically disrupts the β-catenin/TCF interaction is a difficult task (Daniels et al., 2001; Lepourcelet et al., 2004). On the contrary, our mapping and specificity results indicate that the β-catenin/Lgs interaction site could be targeted without interfering with the binding of β-catenin to APC and E-cadherin. Moreover, our analysis shows that genetic disruption of the Arm/Lgs interaction leads to severely reduced Wg signalling, suggesting that the protein-protein interaction between β-catenin and Lgs may provide an attractive target for therapeutic intervention.

We thank W. Birchmeier, R. Kemler and J. P. von Kries for plasmids; I. Duncan and T. Uemura for antibodies; M. Peifer for the *arm*^{2a9} allele; A. Erkner, D. Frey, T. Kramps and D. Nellen for help; and G. Lettre as well as members of our laboratory for comments on the manuscript. This project was supported by the Swiss National Science Foundation and the Kanton of Zürich.

References

Aberle, H., Butz, S., Stappert, J., Weissig, H., Kemler, R. and Hoschuetzky, H. (1994). Assembly of the cadherin-catenin complex in vitro with recombinant proteins. *J. Cell. Sci.* **107**, 3655-3663.

Bartel, P. L. and Fields, S. (1997). In *The Yeast Two-hybrid System*, Chapter 3. New York, NY: Oxford University Press.

Basler, K. and Struhl, G. (1994). Compartment boundaries and the control of Drosophila limb patterning by hedgehog protein. *Nature* **368**, 208-214.

Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R. and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* **382**, 638-642.

Bejsovec, A. and Wieschaus, E. (1993). Segment polarity gene interaction modulate epidermal patterning in *Drosophila* embryos. *Development* **119**, 501-517.

Belenkaya, T. Y., Han, C., Standley, H. J., Lin, X., Houston, D. W., Heasman, J. and Lin, X. (2002). Pygopus encodes a nuclear protein essential for wingless/Wnt signaling. *Development* **129**, 4089-4101.

Bronner, G., Chu-LaGraff, Q., Doe, C. Q., Cohen, B., Weigel, D., Taubert, H. and Jackle, H. (1994). Sp1/egr-like zinc-finger protein required for endoderm specification and germ-layer formation in Drosophila. *Nature* **369**, 664-668.

Brunner, E., Peter, O., Schweizer, L. and Basler, K. (1997). Pangolin encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in Drosophila. *Nature* **385**, 829-833.

Clontech (2001). *Yeast Protocols Handbook*. Palo Alto, CA: Clontech Laboratories.

Dahmann, C. and Basler, K. (2000). Opposing transcriptional outputs of Hedgehog signaling and engrailed control compartmental cell sorting at the Drosophila A/P boundary. *Cell* **100**, 411-422.

Daniels, D. L., Eklof Spink, E. and Weis, W. I. (2001). beta-catenin: molecular plasticity and drug design. *Trends Biochem. Sci.* **26**, 672-678.

Diaz-Benjumea, F. J. and Cohen, S. M. (1995). Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the Drosophila wing. *Development* **121**, 4215-4225.

Eklof Spink, K., Fridman, S. G. and Weis, W. I. (2001). Molecular mechanisms of beta-catenin recognition by adenomatous polyposis coli revealed by the structure of an APC-beta-catenin complex. *EMBO J.* **20**, 6203-6212.

Ferlay, J., Bray, F., Pisani, P. and Parkin, D. M. (2001). *GLOBOCAN 2000: Cancer Incidence, Mortality and Prevalence Worldwide*, Version 1.0. IARC CancerBase No. 5. Lyon, France: IARC Press.

Giles, R. H., van Es, J. H. and Clevers, H. (2003). Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim. Biophys. Acta* **1653**, 1-24.

Graham, T. A., Weaver, C., Mao, F., Kimelman, D. and Xu, W. (2000). Crystal structure of a beta-catenin/Tcf complex. *Cell* **103**, 885-896.

Graham, T. A., Ferkey, D. M., Mao, F., Kimelman, D. and Xu, W. (2001). Tcf4 can specifically recognize beta-catenin using alternative conformations. *Nat. Struct. Biol.* **8**, 1048-1052.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B. and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. *Science* **281**, 1509-1512.

Huber, A. H. and Weis, W. I. (2001). The structure of the beta-catenin/E-cadherin complex and the molecular basis of diverse ligand recognition by beta-catenin. *Cell* **105**, 391-402.

Huber, A. H., Nelson, W. J. and Weis, W. I. (1997). Three-dimensional structure of the armadillo repeat region of beta-catenin. *Cell* **90**, 871-882.

Hulsken, J., Birchmeier, W. and Behrens, J. (1994). E-cadherin and APC

compete for the interaction with beta-catenin and the cytoskeleton. *J. Cell Biol.* **127**, 2061-2069.

Kinzler, K. W. and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. *Cell* **87**, 159-170.

Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC –/– colon carcinoma. *Science* **275**, 1784-1787.

Kramps, T., Peter, O., Brunner, E., Nellen, D., Froesch, B., Chatterjee, S., Murone, M., Zullig, S. and Basler, K. (2002). Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. *Cell* **109**, 47-60.

Lepourcelet, M., Chen, Y. N., France, D. S., Wang, H., Crews, P., Petersen, F., Bruseo, C., Wood, A. W. and Shivdasani, R. A. (2004). Small-molecule antagonists of the oncogenic Tcf/beta-catenin protein complex. *Cancer Cell* **5**, 91-102.

Miller, J. R. and Moon, R. T. (1997). Analysis of the signaling activities of localization mutants of beta-catenin during axis specification in *Xenopus*. *J. Cell Biol.* **139**, 229-243.

Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K. W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* **275**, 1787-1790.

Nüsslein-Volhard, C., Wieschaus, E. and Kludling, H. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. *Rouxs Arch. Dev. Biol.* **193**, 267-282.

Omer, C. A., Miller, P. J., Diehl, R. E. and Kral, A. M. (1999). Identification of Tcf4 residues involved in high-affinity beta-catenin binding. *Biochem. Biophys. Res. Commun.* **256**, 584-590.

Pai, L. M., Orsulic, S., Bejsovec, A. and Peifer, M. (1997). Negative regulation of Armadillo, a Wingless effector in Drosophila. *Development* **124**, 2255-2266.

Parker, D. S., Jemison, J. and Cadigan, K. M. (2002). Pygopus, a nuclear PHD-finger protein required for Wingless signaling in Drosophila. *Development* **129**, 2565-2576.

Peifer, M. and Wieschaus, E. (1990). The segment polarity gene armadillo encodes a functionally modular protein that is the Drosophila homolog of human plakoglobin. *Cell* **63**, 1167-1178.

Peifer, M., Orsulic, S., Sweeton, D. and Wieschaus, E. (1993). A role for the Drosophila segment polarity gene armadillo in cell adhesion and cytoskeletal integrity during oogenesis. *Development* **118**, 1191-1207.

Peifer, M., Berg, S. and Reynolds, A. B. (1994). A repeating amino acid motif shared by proteins with diverse cellular roles. *Cell* **76**, 789-791.

Pokutta, S. and Weis, W. I. (2002). The cytoplasmic face of cell contact sites. *Curr. Opin. Struct. Biol.* **12**, 255-262.

Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S. C., Grosschedl, R. and Bienz, M. (1997). LEF-1, a nuclear factor coordinating signaling inputs from wingless and decapentaplegic. *Cell* **88**, 777-787.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R. and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc. Natl. Acad. Sci. USA* **96**, 5522-5527.

Sparks, A. B., Morin, P. J., Vogelstein, B. and Kinzler, K. W. (1998). Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. *Cancer Res.* **58**, 1509-1512.

Thompson, B., Townsley, F., Rosin-Arbesfeld, R., Musisi, H. and Bienz, M. (2002). A new nuclear component of the Wnt signalling pathway. *Nat. Cell. Biol.* **4**, 367-373.

Tolwinski, N. S. and Wieschaus, E. (2001). Armadillo nuclear import is regulated by cytoplasmic anchor Axin and nuclear anchor dTCF/Pan. *Development* **128**, 2107-2117.

Tolwinski, N. S. and Wieschaus, E. (2004). A Nuclear Function for Armadillo/beta-Catenin. *PloS Biol.* **2**, 1-8.

Uemura, T., Oda, H., Kraut, R., Hayashi, S., Kotaoka, Y. and Takeichi, M. (1996). Zygotic Drosophila E-cadherin expression is required for processes of dynamic epithelial cell rearrangement in the Drosophila embryo. *Genes Dev.* **10**, 659-671.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A. et al. (1997). Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell* **88**, 789-799.

van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A. P. et al. (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **111**, 241-250.

von Kries, J. P., Winbeck, G., Asbrand, C., Schwarz-Romond, T., Sochnikova, N., Dell'Oro, A., Behrens, J. and Birchmeier, W. (2000). Hot spots in beta-catenin for interactions with LEF-1, conductin and APC. *Nat. Struct. Biol.* **7**, 800-807.

Wodarz, A. and Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol.* **14**, 59-88.

Wodarz, A., Hinz, U., Engelbert, M. and Knust, E. (1995). Expression of crumbs confers apical character on plasma membrane domain of ectodermal epithelia of Drosophila. *Cell* **82**, 67-76.

Xing, Y., Clements, W. K., Kimelman, D. and Xu, W. (2003). Crystal structure of a beta-catenin/axin complex suggests a mechanism for the beta-catenin destruction complex. *Genes Dev.* **17**, 2753-2764.

Zecca, M., Basler, K. and Struhl, G. (1996). Direct and long-range action of a wingless morphogen gradient. *Cell* **87**, 833-844.

2.1.1 Unpublished experiments supplemental to 'Identification and in vivo role of the Armadillo-Legless interaction' (Hoffmans and Basler, 2004).

In Hoffmans and Basler (2004) we showed that amino acid D164 of β-catenin and Armadillo (Arm) is important for BCL9 and Legless (Lgs) binding, respectively. Furthermore, Arm which can no longer bind Lgs was severely comprised in its signalling function *in vivo*. We wanted to confirm these results in an *in vitro* signalling assay in both *Drosophila* and mammalian tissue culture cells. To test the signalling dependence of Arm for Lgs binding, the activities of Arm^{S10}-wt and Arm^{S10}-D164A were compared in *Drosophila* S2 cells. Arm^{S10}-wt is a very potent activator of the *wf-luciferase* reporter (Hoffmans et al., 2005), whereas Arm^{S10}-D164A is not (Figure IA). This matches very well with the *in vivo* results, where overexpression of Arm^{S10}-wt leads to gain of function phenotype (naked cuticle) while Arm^{S10}-D164A does not.

Similar results were obtained for β-catenin in 293T cells. In order to bypass potential interference of the endogenous protein in this system, we made use of a *UAS-luciferase* reporter (Hoffmans et al., 2005). β-catenin was fused to the DNA binding domain of Gal4 (G4DBD-β-catenin) and led to strong transcriptional activation of the reporter. In contrast, the D164A version was severely impaired in this activation (Figure IB). This suggests that in both *Drosophila* as well as mammalian tissue culture cells Arm and β-catenin are dependent on Lgs and BCL9 binding for transcriptional activation.

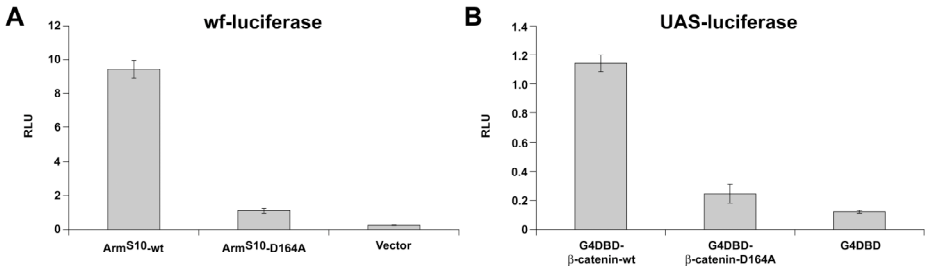

Figure I: Arm and β-catenin depend on Lgs and BCL9 binding for signalling.

(A) *Drosophila* S2 cells were transfected in 24 well-plates with 4 µg DNA per 3 wells (200 ng *wf-luciferase*, 200 ng *actin5c-renilla*, 200 ng *tubulinα1-Gal4*, 3.2 µg empty vector and 200 ng of either *UAS-*Arm^{S10} construct). Whereas Arm^{S10}-wt can strongly activate the reporter, Arm^{S10}-D164A cannot as measured after 48 hours.

(B) Human embryonic kidney 293T cells were transfected in 12-well plates with 1.5 µg of DNA per 3 wells (500 ng *UAS-luciferase*, 100 ng *pRL-TK* (Promega), 400 ng empty vector and 500 ng of either *G4DBD* constructs). Constitutively active (S33Y) mouse β-catenin bound to the Gal4 DNA binding domain (G4DBD-β-catenin-wt) causes a robust activation of the UAS-luciferase reporter as measured after 48 hours. However, the BCL9 binding mutant (G4DBD-β-catenin-D164A) shows strongly reduced activity.

Error bars represent standard deviations of triplicates. Expression levels among the different Arm and β-catenin constructs were equal as measured by western blotting (data not shown).

2.2 Pygopus and Legless provide essential transcriptional coactivator functions to Armadillo/β-catenin

Raymond Hoffmans[1], Reto Städeli[1] and Konrad Basler (2005)
Curr Biol. 15, 1207-1211.

[1] These authors contributed equally to this work and should be considered co-first authors.

Current Biology, Vol. 15, 1207–1211, July 12, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.cub.2005.05.054

Pygopus and Legless Provide Essential Transcriptional Coactivator Functions to Armadillo/β-Catenin

Raymond Hoffmans,[1] Reto Städeli,[1] and Konrad Basler*
Institut für Molekularbiologie
Universität Zürich
Winterthurerstrasse 190
CH-8057 Zürich
Switzerland

Summary

Wnt signaling controls important aspects of animal development, and its deregulation has been causally linked to cancer. Transduction of Wnt signals entails the association of β-catenin with nuclear TCF DNA binding proteins and the subsequent activation of target genes. The transcriptional activity of Armadillo (Arm, the *Drosophila* β-catenin homolog) largely depends on two recently discovered components, Legless (Lgs) and Pygopus (Pygo). Lgs functions as an adaptor between Arm/β-catenin and Pygo, but different mechanisms have been proposed as to how Arm/β-catenin is controlled by Lgs and Pygo. Although Lgs and Pygo were originally thought to serve as nuclear cofactors for Arm/β-catenin to enhance its transactivation capacity, a recent analysis argued that they function instead to target Arm/β-catenin to the nucleus. Here, we used genetic assays in cultured cells and in vivo to discriminate between the two paradigms. Regardless of the measures taken to maintain the nuclear presence of Arm/β-catenin, a transcriptional-activation function of Pygo could not be bypassed. Our findings therefore indicate that Arm/β-catenin depends on Lgs and Pygo primarily for its transcriptional output rather than for its nuclear import.

Results and Discussion

Wingless/Int (Wnt) signals are secreted glycoproteins controlling many fundamental processes during animal development [1]. Whereas several responses to Wnt ligands appear to entail direct cytoplasmic responses organizing planar cell polarity and organ morphogenesis [2, 3], a significant fraction of Wnt responses concern transcriptional changes in the nucleus [4]. This latter aspect of Wnt-signal transduction is mediated by β-catenin and is often referred to as "canonical" or β-catenin-dependent Wnt signaling. The canonical Wnt pathway plays important roles in embryonic-cell-fate determination, and its constitutive activation is oncogenic in several adult mammalian tissues, most notably in the intestinal epithelium [5, 6]. Hence, it is of prime interest to understand how β-catenin activity can upregulate transcription of Wnt target genes. Although cytoplasmic β-catenin was originally discovered through its role in cell adhesion, a large body of evidence indicates that it is degraded in the absence of a Wnt signal but stabilized in its presence. As a consequence, β-catenin can sufficiently accumulate, translocate to the nucleus, and be directed to Wnt target genes by associating with DNA-binding TCF/LEF proteins (reviewed in [7, 8]). However, it is less clear how a cell-adhesion component, relocated to the nucleus, can promote and sustain the transcriptional activity of these targets.

Using genetic assays in *Drosophila*, we have recently identified a presumptive adaptor protein, Legless (Lgs) [9], that binds to β-catenin and its *Drosophila* homolog, Armadillo (Arm), as well as to the nuclear protein Pygopus (Pygo) [9–12]. On the basis of biochemical and phenotypic analysis, we proposed that nuclear β-catenin/Arm assembles a quaternary complex, consisting of TCF, β-catenin, Lgs, and Pygo, in which Pygo serves as a transcriptional activator to induce and/or maintain the transcription of Wnt/Wg target genes [9]. Alternatively, however, the requirement for Lgs and Pygo in Wnt/Wg signaling could be attributed to a role in targeting and retaining β-catenin in the nucleus, increasing its net nuclear concentration and, hence, its activity. This latter view has recently gained recognition [13] and experimental support [14] by a cell-biological analysis of these components. Here, we set out to address the mechanistic role of Pygo by subjecting the two models to three different tests and come, in each case, to the conclusion that Pygo functions mainly in the transcriptional output of β-catenin.

In the first approach, we examined the consequences of disrupting the molecular interaction between β-catenin and Lgs. We have recently identified β-catenin/Arm amino acid residues required for Lgs binding and observed that mutant β-catenin forms lacking these residues are severely compromised in their signaling activity [15]. This reduction in activity could be caused either by a failure of β-catenin/Arm to recruit the "transcriptional mediator" Pygo or by a reduced (as a result of diminished nuclear anchoring) nuclear-cytoplasmic ratio of β-catenin/Arm. We repeated an experiment in which N-terminally truncated and therefore constitutively active forms of Arm, Arm^{S10}-wt and Arm^{S10}-D164A (differing solely in one critical amino acid residue necessary for Lgs binding), were expressed in the embryonic epidermis of *Drosophila* [15, 16]. Expression of Arm^{S10}-wt suppressed denticle formation (Figure 1B)—a read-out for a gain of Wg signaling activity—whereas the D164A mutation, which impairs binding to Lgs, efficiently abolished this gain-of-function activity (Figure 1C and [15]). When subjected to an immunohistochemical analysis, however, the two genotypes visually differed neither in amount nor subcellular localization of the Arm^{S10} proteins (Figures 1A′–1C′). We further used the D164A mutation in a cellular assay in which a constitutively active form of β-catenin (S33Y) was tethered to the enhancer of a reporter gene by the DNA binding domain of Gal4. Whereas β-catenin caused strong transcriptional acti-

*Correspondence: basler@molbio.unizh.ch
[1]These authors contributed equally to this work and should be considered co-first authors.

Current Biology
1208

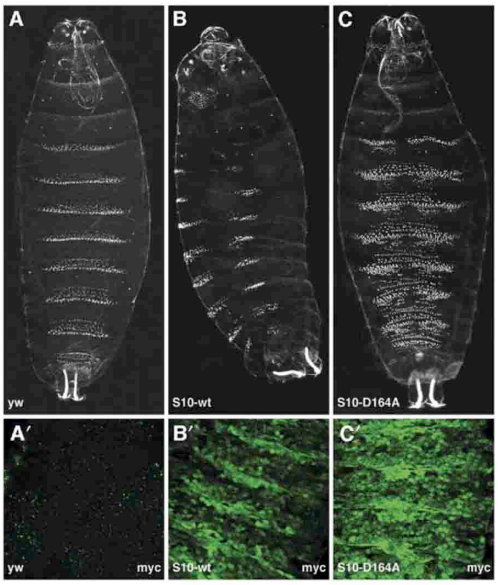

Figure 1. Constitutively Active Forms of Arm Depend on Lgs Binding for Signaling But Not for Localization

(A–C) Cuticles of embryos with different genotypes are shown. (A) Wild-type embryos show a repeated pattern of denticle belts on their ventral surface. (B) Ubiquitous expression of a constitutively active form of Arm (Arm^{S10}-wt) results in a naked-cuticle phenotype, a hallmark of ectopic Wg signaling activity [16, 27]. (C) Ubiquitous expression of a constitutively active Arm form impaired for Lgs binding (Arm^{S10}-D164A) no longer causes a naked-cuticle phenotype; indeed, a slight dominant-negative effect can be observed (ectopic denticles between denticle belts).

(A′–C′) Expression of transgene-derived Arm proteins was revealed by confocal microscopy with an α-myc antibody. (A′) No staining is seen in wild-type embryos. (B′, C′) Arm^{S10} proteins are present in the nucleus and also in the cytoplasm. The localization of Arm^{S10}-D164A cannot be distinguished from that of Arm^{S10}-wt.

All *arm* transgenes in these experiments contain a Myc epitope in their C-terminal region [15, 16] and were controlled by a UAS-promoter driven by *daughterless-Gal4* [28].

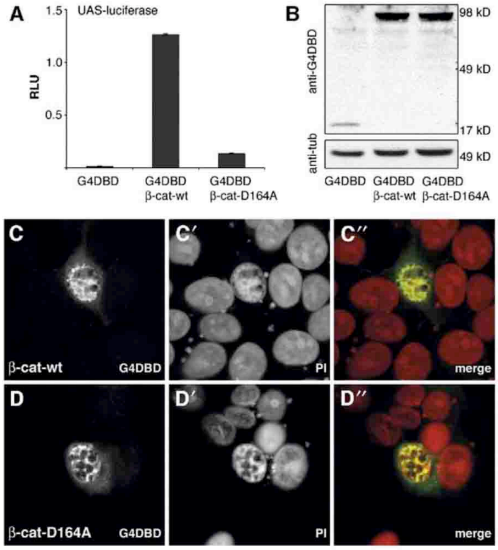

Figure 2. The D164A Mutation, Which Prevents Lgs Binding, Severely Reduces the Transcriptional Activity of β-Catenin without Affecting Subcellular Localization

(A) Relative reporter-gene activities induced by DNA-tethered mouse β-catenin-wt and β-catenin-D164A assayed in 293T cells. Constitutively active β-catenin (S33Y) bound to the Gal4 DNA binding domain (G4DBD-S33Y-β-cat-wt) causes a strong transcriptional activation of the UAS-luciferase reporter, whereas the D164A mutant form has strongly reduced activity. Error bars represent standard deviations of triplicates.

(B) Western blot with 293T cell lysates demonstrates that both forms of β-catenin proteins are expressed at equivalent levels.

(C and D) Immunostaining of transfected 293T cells shows no significant differences in subcellular localization between G4DBD-S33Y-β-cat-wt and G4DBD-S33Y-β-cat-D164A proteins.

(C′ and D′) Propidium iodide (PI) staining to mark cell nuclei.

(C″ and D″) Merge of the panels to the left, with PI staining in red, G4DBD in green.

We obtained essentially the same results with Gal4-β-catenin fusion proteins that additionally contained an N-terminal nuclear-localization signal (NLS-G4DBD-S33Y-β-cat-wt and NLS-G4DBD-S33Y-β-cat-D164A).

vation, the D164A form lost this activity almost completely (Figure 2A). Importantly, however, both forms were expressed at equivalent levels in human cells (Figure 2B) and did not differ in their ability to localize in nuclei (Figures 2C and 2D). Because Lgs mediates the binding of β-catenin to Pygo [9, 17], we interpret these results as evidence that a failure of Arm/β-catenin to recruit Pygo impedes the transcriptional activity of the former despite the fact that it is nuclearly localized.

A second test was devised on the assumption that Lgs appears to function merely as an adaptor between Arm/β-catenin and Pygo, thereby linking Arm/β-catenin either to a transcriptional activator or a nuclear anchor. Such a passive role for Lgs can be inferred from the observations that Lgs is dependent on Pygo both for its signaling activity [9] and for its nuclear localization [14]. If the main role of Lgs would be to link Arm/β-catenin to the constitutively nuclear anchor Pygo, it should gain functional independence of Pygo when bestowed with a nuclear-localization signal (NLS). We therefore modified Lgs by replacing a C-terminal portion with sequences of a green fluorescent protein (LgsN-eGFP) and adding the NLS of SV40 large T-antigen N-terminally (NLS-LgsN-eGFP). These altered forms of Lgs were examined for their subcellular distribution and signaling function. The addition of a single NLS effectively conferred nuclear localization, as assessed in transfected cells (Figures 3A–3D). When tested for their signaling capacity in *Drosophila* S2 cells, LgsN and NLS-LgsN were found to be equally active in rescuing the RNAi-mediated knockdown of endogenous Lgs (Figure 3E). However, these two forms of Lgs were equally inactive in rescuing the knockdown of endogenous Pygo. Consistent with this result, we also found that the Lgs-rescuing activity of NLS-LgsN still depends on the HD1 domain [9], through which it binds Pygo (Figure

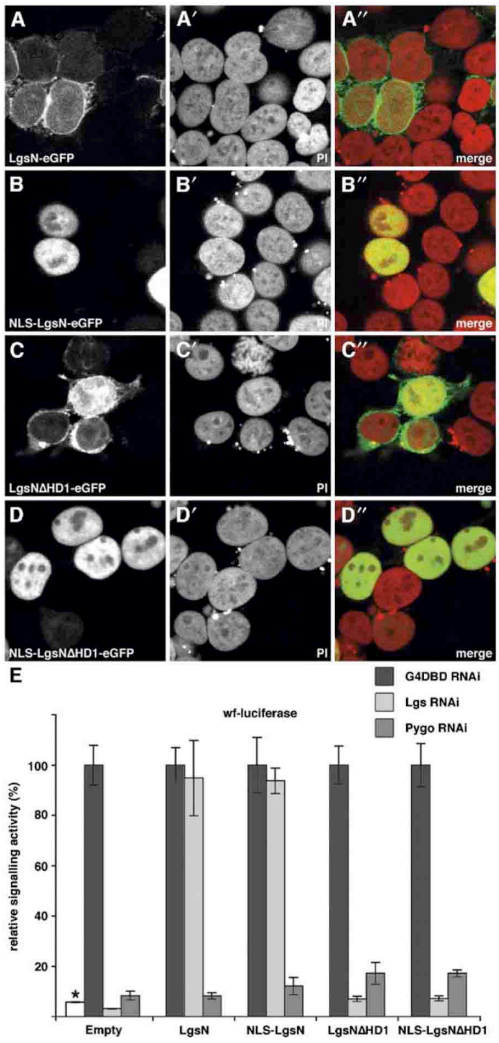

Figure 3. Nuclearly Targeted LgsN Can Rescue the Knockdown of *lgs* But Not of *pygo* in a Cell-Based Wg Signaling Assay

(A–D) Localization of the different forms of Lgs in 293T cells. All forms contain eGFP instead of the C-terminal amino acids 783 to 1464. An even shorter version of Lgs (amino acids 1–583) has been shown to rescue *lgs* mutant animals [9]. Most LgsN and LgsNΔHD1 protein is found in the cytoplasm (except for a small percentage of cells in which LgsNΔHD1 can be seen all over the cell). NLS-LgsN and NLS-LgsNΔHD1, however, are exclusively nuclear.

(A′–D′) Propidium iodide (PI) staining to mark cell nuclei.

(A″–D″) Merge of the panels to the left, PI staining in red, eGPF in green. The same results were obtained in HeLa cells (data not shown).

(E) Signaling capacities of different forms of Lgs (eGFP-tagged) are shown in the presence or absence of endogenous Lgs and Pygo. LgsN was used here instead of full-length Lgs to allow selective knockdown of endogenous Lgs by means of RNAi directed against the 3′ half of the *lgs* transcript. *Drosophila* S2 cells were transfected with the *wf-luc* reporter construct (see Experimental Procedures), expression plasmids as indicated underneath the bar diagram, and were treated with dsRNA as indicated in the legend. Cells were cocultured with Wg-secreting cells [25], resulting in a 20-fold stimulation of *luciferase* expression. All bars are from cells in the Wg-induced state except the asterisk-marked one, which represents the uninduced state. Rescuing activities of the different forms of Lgs are represented relative to the respective G4DBD negative control RNAi (set to 100%). LgsN can rescue *lgs* RNAi but not *pygo* RNAi. The same was observed for NLS-LgsN. Neither LgsNΔHD1 nor NLS-LgsNΔHD1 can rescue *lgs* or *pygo* RNAi, indicating that the signaling capacities of LgsN and NLS-LgsN depend on Pygo binding. Error bars represent standard deviations of triplicates.

3E). Together, these results indicate that constitutive nuclear targeting of Lgs does not bypass the requirement for Pygo in Wg signaling, suggesting that Pygo must provide a function beyond ensuring availability of Lgs and β-catenin in the nucleus of Wg-transducing cells.

Our third test aimed at assessing the role of the N-terminal homology domain (NHD) of Pygo. *Drosophila* Pygo and its two mammalian homologs, Pygo1 and Pygo2, share—in addition to their C-terminal plant homology domain (PHD) finger domain, through which they bind Lgs—a short N-terminally located sequence of amino acids [9, 12]. On the basis of the conservation of Pygo function and absence of further common domains, the NHD was proposed to serve as transactivation domain [9]. We first confirmed that the NHD core domain (amino acids 91 to 101) is not required for nuclear localization of Pygo because neither the deletion of the core nor the change of a conserved and functionally required amino acid (F99A) affected the nuclear localization of Pygo in cultured cells (data not shown), consistent with the findings of Townsley et al. [18]. Importantly, these alterations also had no discernible effect on the capacity of Pygo to bind Lgs, as shown for Pygo-F99A in Figure 4A. If Pygo and Lgs primarily function to target Arm/β-catenin to the nucleus, then NHD mutations should not seriously affect Wnt/Wg signaling. We found, however, that Pygo-F99A—in contrast to wild-type Pygo—failed to rescue Pygo function in cultured cells and in vivo (R.S. and K.B., unpublished data). We then replaced the endogenous *pygo* gene with a genomic *pygo-F99A* transgene in vivo, and we observed that both mutant and wild-type Pygo proteins were expressed at comparable levels without detectable differences in nuclear-cytoplasmic distribution (Figures 4B–4D). The most explicit argument for a role of the NHD in transactivation was obtained by analyzing mutant clones of imaginal cells in which either the *pygo-wt* or the *pygo-F99A* transgenes were the only source of full-length Pygo protein (Figures 4E–4G). Both transgenes rescued Lgs nuclear localization in the mutant clones to a similar extent; however, *pygo-F99A*—but not *pygo-wt*—showed severely reduced transcription of the Wg target gene *senseless* [19]. Because Pygo protein bearing a mutant NHD retains the capacity to localize Lgs (and, by inference, Arm), we infer that the key function of the Pygo NHD is to confer transcriptional activity to Arm.

In summary, we have tested the function of Lgs and Pygo in β-catenin-dependent Wnt/Wg signaling by devising experiments that separate a role in transcriptional ac-

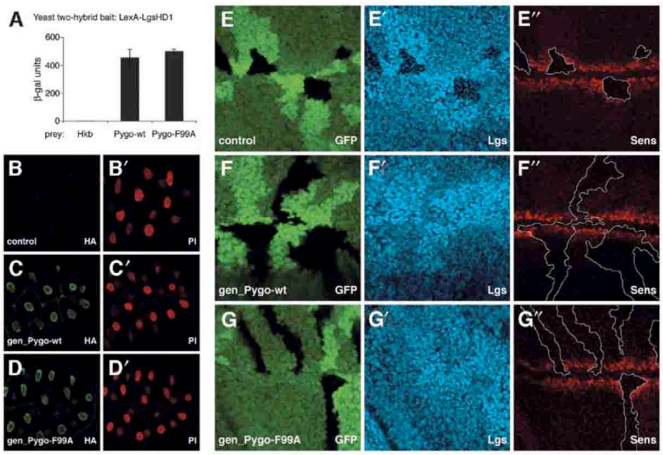

Figure 4. A Single-Point Mutation in the Pygo NHD Affects Neither Lgs Binding Nor Nuclear Localization, But Severely Reduces Its Signaling Ability

(A) A quantitative yeast two-hybrid assay confirms that dPygo-wt and dPygo-F99A both strongly bind to LgsHD1 but not to the negative control Huckebein (Hkb), a transcription factor [29] that plays no role in Wnt/Wg signaling. Error bars represent standard deviations of triplicates.

(B–D) Immunostaining of *Drosophila* salivary glands demonstrates that dPygo-wt and dPygo-F99A localize to the nucleus.

(B′–D′) Propidium iodide (PI) staining to mark cell nuclei.

(E–G) *Drosophila* wing imaginal discs containing cell clones from which endogenous *pygo* function has been eliminated. The clones are marked by the absence of GFP expression (GFP shown in green) and assayed for Lgs distribution (blue) and Senseless (Sens) expression (red). (E) In discs expressing no *pygo* transgene, the removal of *pygo* activity causes cytoplasmic localization of Lgs and a severe reduction of Sens expression. (F) A genomic transgene encoding dPygo-wt partly rescues nuclear Lgs localization and Sens expression in *pygo* mutant tissue. (G) dPygo-F99A can only rescue Lgs localization, but not restore Sens expression in *pygo* clones.

tivation of targets from a role in nuclear targeting or retention of Arm/β-catenin. In all three situations examined, the transcriptional output of Arm/β-catenin depended on Pygo activity despite measures to grant Arm/β-catenin such alleged nuclear retention. When Arm/β-catenin was tethered directly to DNA via the Gal4 DNA binding domain, or when Lgs was endowed with an NLS of its own, Arm/β-catenin activity was still dependent on the recruitment of Pygo. Likewise, in vivo, when the nuclear retention activity of Pygo was left intact, Arm was not able to transduce Wg and activate target genes without the Pygo NHD. Although we cannot rule out that Lgs and Pygo function as a nuclear anchor for β-catenin, our results collectively argue that the primary requirement for the two Arm/β-catenin partners must be attributed to a transcriptional role that allows Arm/β-catenin to activate and/or sustain the expression of Wnt/Wg target genes. Although we presently lack information on the biochemical nature of this transactivation activity, it is tempting to assume that it involves the NHD-mediated recruitment of a chromatin-modification complex or of factors mediating transcription initiation or elongation.

Experimental Procedures

Plasmid Constructs

For the *wf-luc* reporter plasmid, a 2.2 kb fragment of the *wingful/Notum* gene ([20, 21]; 4099 to 1866 bp upstream of ATG) was amplified by polymerase chain reaction (PCR) and inserted into the pGL3 firefly luciferase reporter (Promega). The minimal *SV40* promoter was replaced by a minimal *hsp70* promoter (A. Smith, M. Kuster, R.S., and K.B., unpublished data). The *UAS-luc* reporter plasmid used in 293T cells was generated by inserting the five tandem-arrayed optimized Gal4 binding sites from pUAST [22] into a modified pGL3 vector, where the minimal *SV40* promoter was replaced by a minimal *cFos* promoter. pcDNA3.1 (Invitrogen) or pPacPL [23] vectors were used for expression of cDNAs in mammalian cells or S2 cells, respectively. The LgsN constructs contain amino acids 1–782. In the ΔHD1 constructs, amino acids 318–345 are replaced by a BglII site. For simplicity, we use the amino acid numbering of *Drosophila* Pygo for both human and *Drosophila* Pygo throughout the text. dPygo-F99 would correspond to hPygo2-F78.

Antibodies

The following antibodies were used: rabbit anti-Gal4DBD (SC-577, Santa Cruz), rabbit anti-Myc (A14, Santa Cruz), rat anti-HA (3F10, Roche), mouse anti-Tubulin (B-5-1-2, Sigma), rabbit anti-Lgs [9], guinea pig anti-Senseless [19], goat anti-rabbit conjugated with Alexa 488 (Molecular Probes), goat anti-rat conjugated with fluorescein isothiocyanate (FITC) (Jackson Labs), goat anti-rabbit-HRP (Jackson Labs), goat anti-rabbit conjugated with Cy5 (Jackson Labs), and goat anti-guinea pig conjugated with Alexa 568 (Molecular Probes).

dsRNA Production

Templates for G4DBD, GFP, Lgs, and Pygo double-stranded RNA (dsRNA) synthesis were generated by PCR with the following primers containing 5′ T7 promoter tails:

fG4DBD 5′-T7CTACTGTCTTCTATCGAACAAG-3′, rG4DBD 5′-T7ATACAGTCAACTGTCTTTGAC-3′, product length: 0.43kb;
fGFP 5′-T7CTTTTCACTGGAGTTGTCC-3′, rGFP 5′-T7ATCCATGCCATGTGTAATCC-3′, product length: 0.68kb;
fLgs 5′-T7GGCATGCGTCCACATGCC-3′, rLgs 5′-T7ATTTGTTGACAAAGAACGTTG-3′, product length: 0.58kb;
fPygo 5′-T7TGGTGATGCGGATGTCC-3′, rPygo 5′-T7TCCATGTCCACGTCAGTGC-3′, product length 0.61kb;

dsRNA was then synthesized from these templates with the Ambion Megascript kit. For RNAi in S2 cells [24], 1 μg dsRNA was used per well (96-well plate).

Cell Culture

All transfections were performed with polyethylenimine (PEI, Polysciences). S2-cell transfections were done in 96-well plates with a total of 500 ng DNA per 3 wells (50 ng *wf-luc*, 100 ng *actin5c-renilla*, 50 ng *tubulinα1-dfrizzled2*, 50 ng rescue constructs, and 250 ng empty vector). The Wg signaling pathway was induced 48 hr after transfection by the addition of heat-shocked S2 cells stably transfected with Wg cDNA under the control of a heat-shock promoter as described [25]. 293T cell transfections were performed in 12-well plates with a total of 1.5 ug DNA per 3 wells (500 ng *UAS-luc*, 100 ng pRL-TK (Promega), 500 ng Gal4DBD constructs, and 400 ng of empty vector). Luciferase activities were determined after 72

hr for S2 cells and after 48 hr for 293T cells via the Dual-Luciferase Assay System (Promega).

Immunohistochemistry

For embryonic stainings, embryos were dechorionated in bleach and fixed for 30 min at the interphase of a heptane/8% formaldehyde in PEM (0.1 M Pipes, 1 mM EGTA, and 2 mM $MgSO_4$) solution. The aqueous phase was removed, and an equal amount of methanol was added to devitellinize the embryos. Mutant clones in wing imaginal discs were generated by crossing FRT82 $pygo^{S130}$/TM6b carrying either genomic Pygo-wt or F99A to hsp70-flp; FRT82 ubi-GFP/TM6b. Seventy-two hours after egg laying, larvae were heat shocked at 37.5°C for 45 min. Late third-instar larvae were dissected, and imaginal discs and salivary glands were fixed and stained by standard techniques.

293T cells were fixed in 4% paraformaldehyde 48 hr after transfection.

Yeast Two-Hybrid Assays

The yeast two-hybrid system as described in chapter 3 of Bartel and Fields [26] was used. Interactions between proteins were measured with the quantitative "Liquid Culture Assay Using ONPG as Substrate" (Clontech, Yeast Protocols Handbook, http://www.clontech.com/clontech/techinfo/manuals).

Acknowledgments

We thank G. Hausmann for invaluable advice, H. Bellen for antibodies, and A. Hajnal and members of our laboratory for comments on the manuscript. This work was supported by the Swiss National Science Foundation and the Kanton of Zürich.

Received: April 13, 2005
Revised: May 17, 2005
Accepted: May 17, 2005
Published: July 12, 2005

References

1. Wodarz, A., and Nusse, R. (1998). Mechanisms of Wnt signaling in development. Annu. Rev. Cell Dev. Biol. *14*, 59–88.
2. Strutt, D. (2003). Frizzled signalling and cell polarisation in Drosophila and vertebrates. Development *130*, 4501–4513.
3. Veeman, M.T., Axelrod, J.D., and Moon, R.T. (2003). A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. Dev. Cell *5*, 367–377.
4. Logan, C.Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. *20*, 781–810.
5. Polakis, P. (2000). Wnt signaling and cancer. Genes Dev. *14*, 1837–1851.
6. Sancho, E., Batlle, E., and Clevers, H. (2004). Signaling pathways in intestinal development and cancer. Annu. Rev. Cell Dev. Biol. *20*, 695–723.
7. Peifer, M., and Polakis, P. (2000). Wnt signaling in oncogenesis and embryogenesis—a look outside the nucleus. Science *287*, 1606–1609.
8. Hurlstone, A., and Clevers, H. (2002). T-cell factors: Turn-ons and turn-offs. EMBO J. *21*, 2303–2311.
9. Kramps, T., Peter, O., Brunner, E., Nellen, D., Froesch, B., Chatterjee, S., Murone, M., Züllig, S., and Basler, K. (2002). Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. Cell *109*, 47–60.
10. Belenkaya, T.Y., Han, C., Standley, H.J., Lin, X., Houston, D.W., Heasman, J., and Lin, X. (2002). Pygopus encodes a nuclear protein essential for wingless/Wnt signaling. Development *129*, 4089–4101.
11. Parker, D.S., Jemison, J., and Cadigan, K.M. (2002). Pygopus, a nuclear PHD-finger protein required for Wingless signaling in Drosophila. Development *129*, 2565–2576.
12. Thompson, B., Townsley, F., Rosin-Arbesfeld, R., Musisi, H., and Bienz, M. (2002). A new nuclear component of the Wnt signalling pathway. Nat. Cell Biol. *4*, 367–373.
13. Tolwinski, N.S., and Wieschaus, E. (2004). A nuclear escort for beta-catenin. Nat. Cell Biol. *6*, 579–580.
14. Townsley, F.M., Cliffe, A., and Bienz, M. (2004). Pygopus and Legless target Armadillo/beta-catenin to the nucleus to enable its transcriptional co-activator function. Nat. Cell Biol. *6*, 626–633.
15. Hoffmans, R., and Basler, K. (2004). Identification and in vivo role of the Armadillo-Legless interaction. Development *131*, 4393–4400.
16. Pai, L.M., Orsulic, S., Bejsovec, A., and Peifer, M. (1997). Negative regulation of Armadillo, a Wingless effector in Drosophila. Development *124*, 2255–2266.
17. Thompson, B.J. (2004). A complex of Armadillo, Legless, and Pygopus coactivates dTCF to activate wingless target genes. Curr. Biol. *23*, 458–466.
18. Townsley, F.M., Thompson, B., and Bienz, M. (2004). Pygopus residues required for its binding to Legless are critical for transcription and development. J. Biol. Chem. *279*, 5177–5183.
19. Nolo, R., Abbott, L.A., and Bellen, H.J. (2000). Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in Drosophila. Cell *102*, 349–362.
20. Gerlitz, O., and Basler, K. (2002). Wingful, an extracellular feedback inhibitor of Wingless. Genes Dev. *16*, 1055–1059.
21. Giraldez, A.J., Copley, R.R., and Cohen, S.M. (2002). HSPG modification by the secreted enzyme Notum shapes the Wingless morphogen gradient. Dev. Cell *2*, 667–676.
22. Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fate and generating dominant phenotypes. Development *118*, 401–415.
23. Koelle, M., and Hogness, D. (1992). Technical Notes: Plasmid vectors for expressing proteins in tissue culture cells or studying enhancer function. Drosophila Information Newsletter *8*, 401–415.
24. Clemens, J.C., Worby, C.A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B.A., and Dixon, J.E. (2000). Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. Proc. Natl. Acad. Sci. USA *97*, 6499–6503.
25. van Leeuwen, F., Samos, C.H., and Nusse, R. (1994). Biological activity of soluble wingless protein in cultured Drosophila imaginal disc cells. Nature *368*, 342–344.
26. Bartel, P.L., and Fields, S. (1997). The Yeast Two-Hybrid System (New York: Oxford University Press).
27. Noordermeer, J., Johnston, P., Rijsewijk, F., Nusse, R., and Lawrence, P.A. (1992). The consequences of ubiquitous expression of the wingless gene in the Drosophila embryo. Development *116*, 711–719.
28. Wodarz, A., Hinz, U., Engelbert, M., and Knust, E. (1995). Expression of crumbs confers apical character on plasma membrane domain of ectodermal epithelia of Drosophila. Cell *82*, 67–76.
29. Bronner, G., Chu-LaGraff, Q., Doe, C.Q., Cohen, B., Weigel, D., Taubert, H., and Jackle, H. (1994). Sp1/egr-like zinc-finger protein required for endoderm specification and germ-layer formation in Drosophila. Nature *369*, 664–668.

2.2.1 Unpublished experiments supplemental to 'Pygopus and legless provide essential transcriptional coactivator functions to armadillo/β-catenin' (Hoffmans et al, 2005).

Townsley et al. (2004) showed that expression of an Armadillo (Arm) protein with a nuclear localization signal (NLS) can partially 'rescue' the embryonic lawn of denticle phenotype of *legless* (*lgs*) and *pygopus* (*pygo*) mutants. Based on this result, the authors suggested that Lgs and Pygo can function as a nuclear anchor for Arm/β-catenin. We reasoned that if the sole function of Lgs and Pygo is to act as a nuclear anchor then NLS-Arm signalling activity should be completely independent of Lgs or Pygo, since the NLS will make Arm always nuclear. To test our hypothesis, the signalling capacity of different forms of NLS-Arm were compared in *Drosophila* S2 cells in the presence or absence of Lgs and Pygo. Expression of NLS-Arm leads to strong transcriptional activation of the *wf-luciferase* reporter gene. This activation is partially dependent on Lgs and Pygo since RNAi against these proteins results in reduced activation (Figure II). Additionally, NLS-Arm protein which is mutant for Lgs binding (NLS-Arm-D164A) also has reduced activity. This suggests that although NLS-Arm is predominantly nuclear, Lgs and Pygo facilitate extra transcriptional activation to NLS-Arm.

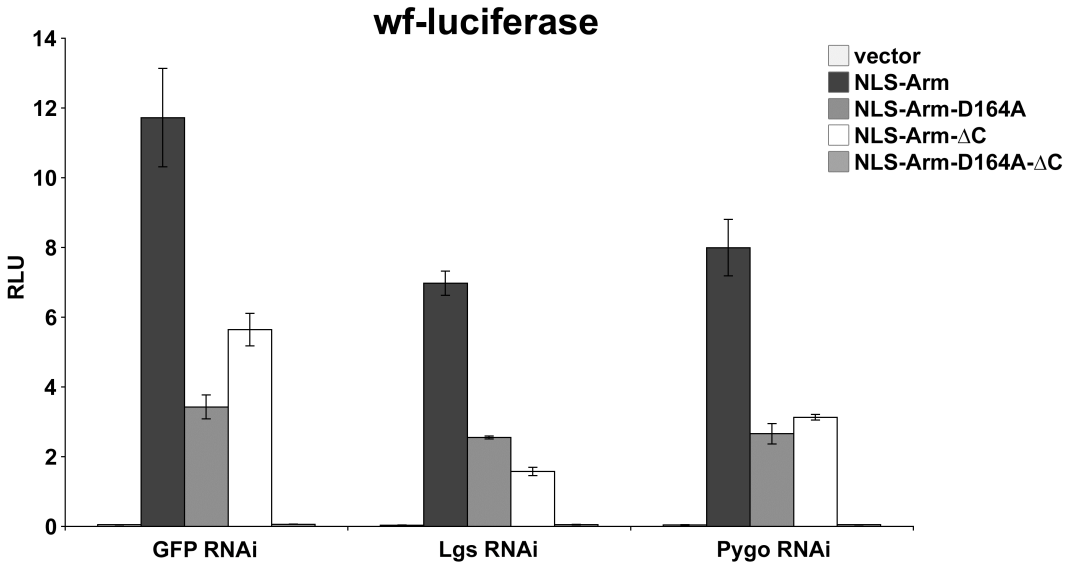

Figure II: NLS-Arm depends on both Lgs binding and the C-terminus of Arm for signalling.
Drosophila S2 cells were transfected in 96 well-plates with 2 µg DNA per 12 wells (200 ng *wf-luciferase*, 400 ng *actin5c-renilla*, 1.2 µg empty vector and 200 ng of either *NLS-Arm* construct) and 1 µg dsRNA per well. Whereas NLS-Arm can strongly activate the reporter, NLS-Arm-D164A is less active as is NLS-Arm-ΔC. A NLS-Arm-D164A-ΔC is completely signalling inactive. Relative reporter gene activity measured after 48 hours. Error bars represent standard deviations of triplicates. In cl8 cells all 4 NLS-Arm constructs are expressed equally well as measured by western blotting (data not shown)

One likely explanation for the ability of NLS-Arm to signal in the absence of Lgs and Pygo is that NLS-Arm can bind other proteins such as CBP/p300, Brg1 and Parafibromin through its C-terminal half (Barker et al., 2001; Hecht et al., 2000; Takemaru and Moon, 2000; Mosimann et al submitted). These proteins have been implicated in chromatin remodeling and transcriptional initiation/elongation (see 1. Introduction for more details). If NLS-Arm signals in the absence of Lgs or Pygo through these C-terminal binding proteins then deletion of the C-terminus should inhibit transcriptional activity. As can be seen in Figure II, deletion of the C-terminus (NLS-Arm-ΔC) reduces the transcriptional output of NLS-Arm. Signalling of NLS-Arm-ΔC can be further inhibited by RNAi against Lgs or Pygo and is completely abrogated in a double mutant for both Lgs binding and the C-terminal deletion (NLS-Arm-D164A-ΔC). This led us to conclude that the signalling of NLS-Arm in *lgs* and *pygo* mutants as seen by Townsley et al. (2004) is dependent on the C-terminus of Arm.

The above described experiment shows that the transactivation activity of Arm depends on Lgs binding and on an intact C-terminal region for signalling. In Chapter 2.5 of this thesis experiments are described that try to decipher what the contribution is of Lgs versus the C-terminal domain for Wg/Wnt signalling.

2.3 The roles of BCL9.2 and tyrosine phosphorylation of Armadillo/β-catenin in Wingless/Wnt signalling.

Raymond Hoffmans and Konrad Basler

in preparation

Introduction

The Armadillo (Arm)/β-catenin protein has a dual function. On the one hand Arm/β-catenin binds α-catenin and the cytoplasmic tail of E-cadherin thereby forming the adherens junctions (Drees et al., 2005; Yamada et al., 2005). On the other hand it is the key effector protein of the Wingless (Wg)/ Wnt signalling pathway. Arm/β-catenin that is not part of the adherens junctions is degraded in the absence of the Wg/Wnt ligand. This degradation is initiated by the binding of Arm/β-catenin to its destruction complex consisting of Adenomatous Polyposis Coli (APC), Axin, Casein Kinase Iα and GSK-3β. The latter two phosphorylate β-catenin, which then is ubiquitinated and sent to the proteasome for degradation (Aberle et al., 1997; Behrens et al., 1998; Ikeda et al., 1998; Kishida et al., 1998; Liu et al., 2002). When the Wg/Wnt ligand is present, it binds the receptor Frizzled which together with Arrow/LRP signals through Dishevelled to inhibit the formation of the destruction complex. As a consequence, Arm/β-catenin accumulates in the cytoplasm and subsequently enters the nucleus where it binds members of the TCF/Lef family of DNA binding transcription factors (Behrens et al., 1996; Brunner et al., 1997; Riese et al., 1997; van de Wetering et al., 1997). In the nucleus β-catenin interacts with additional proteins such as CBP/p300, Parafibromin and Legless (Lgs)/BCL9 (Hecht et al., 2000; Kramps et al., 2002; Takemaru and Moon, 2000, Mosimann et al. unpublished). The latter can also bind with Pygopus (Pygo). While Pygo is necessary for Wg signalling, the role of Pygo in the transcription of Wg/Wnt target genes is still largely unknown (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002; Stadeli and Basler, 2005; Thompson et al., 2002; Thompson, 2004). A chimeric protein in which the Lgs interaction domain of Pygo (PHD) has been replaced by the β-catenin interaction domain of Lgs (HD2) can rescue both *lgs* and *pygo* mutant flies suggesting that the primary function of Lgs is the recruitment of Pygo to Arm/β-catenin (Kramps et al., 2002; Thompson, 2004).

Two recent reports describe the cloning and functional analysis of a second ortholog of Lgs in mammals, BCL9.2. This protein was discovered in two different yeast two-hybrid screens to identify new binding partners of β-catenin (Adachi et al., 2004; Brembeck et al., 2004). Binding of BCL9.2 to β-catenin depends on tyrosine 142 (Y142) of β-catenin, since mutating this amino acid abrogated the binding (Brembeck et al., 2004). In contrast to BCL9, it was shown that BCL9.2 does not depend on Pygo binding for its transactivation activity (Adachi et al., 2004; Brembeck et al., 2004).

In the present study we report that Y142 of β-catenin/Arm is not important for either BCL9, BCL9.2 or Lgs binding. However the BCL9.2-β-catenin interaction is dependent on the aspartic acid 164 (D164) of β-catenin/Arm. We demonstrate that Arm (*Drosophila* homolog of β-catenin) does not depend on Y142 for Wg signalling *in vivo* in *Drosophila*. In addition β-catenin does not depend on Y142 in Wnt signalling assays in mammalian tissue culture cells. We also show that BCL9.2 can functionally replace Lgs both in tissue culture and *in vivo* in *Drosophila* and that this rescue is dependent on Pygo binding.

Results

Tyrosine phosphorylation of Arm/β-catenin is not necessary for BCL9.2 binding

Recently there was a report that put forth a model in which β-catenin Y142 needs to be phosporylated for BCL9.2 binding (Brembeck et al., 2004). We previously identified two amino acids on β-catenin/Arm, D162 and D164, that are necessary for BCL9/Lgs binding (Hoffmans and Basler, 2004). We therefore wanted to know if D164 is also important for BCL9.2 binding and if Y142 has an influence on BCL9/Lgs binding. To test this, β-catenin mutants were used in a quantitative yeast two-hybrid assay for binding to BCL9, BCL9.2 or α-catenin. Mutation of the aspartic acid D164 of β-catenin to alanine (D164A), which is crucial for BCL9-β-catenin interaction, also abolished the binding to BCL9.2. β-catenin-Y142 mutated to alanine (Y142A) did not affect binding of BCL9 but abolished binding of α-catenin (Figure 1A and Aberle et al., 1996; Pokutta and Weis, 2000). Surprisingly, in contrast to previous results Y142A also did not affect binding of BCL9.2 (Brembeck et al., 2004). The aromatic ring of Y142 is important for α-catenin binding (Pokutta and Weis, 2000). By changing Y142 to a phenylalanine (Y142F), the necessity of the free hydroxyl group of Y142 was determined. Y142F had no significant effect on either BCL9, BCL9.2 or α-catenin binding (Figure 1A and Aberle et al., 1996)). In order to mimic phosphorylation, Y142 was replaced by an aspartic or glutamic acid (Y142D and Y142E respectively). This also had no effect on β-catenin binding to BCL9, but BCL9.2 did bind slightly better, whereas Y142D and Y142E abolished α-catenin binding.

Corresponding mutations were introduced in Arm and tested for binding to *Drosophila* homologues and BCL9.2 (Figure 1B). The results were the same as for the mammalian components, with the exception that *Drosophila* α-catenin binds better to Arm-Y142A than α-catenin to β-catenin-Y142A. Together these binding studies suggest that the BCL9, BCL9.2 and Lgs interaction with β-catenin/Arm requires amino acid D164, whereas amino acid Y142 is important for α-catenin binding but not for BCL9, BCL9.2 or Lgs binding.

Arm-Y142 mutants can rescue *arm* null animals

To investigate if an Arm-Y142A mutant has impaired transcriptional activition in *Drosophila* we tested if Arm-Y142A can substitute for wildtype Arm *in vivo* by performing a rescue assay with an *arm* allele. For this purpose we used the arm^{2a9} allele, which has a frameshift mutation in Arm repeat 3 and is presumed to be a null allele (Peifer and Wieschaus, 1990). Hemizygous arm^{2a9} males die as embryos but can be rescued to adults by an Arm-wt transgene driven by the *tubulinα1* promoter without any obvious phenotypes (Table 1 and (Hoffmans and Basler, 2004)). In contrast, a *tubulinα1*-promoter-driven Arm-D164A transgene cannot rescue arm^{2a9} mutants and the males die as embryos or early larvae (Table 1 and (Hoffmans and Basler, 2004). An Arm-Y142A transgene can rescue arm^{2a9} males to adults although not as efficiently as Arm-wt. The rescued males do not show any obvious phenotypes (Table 1).

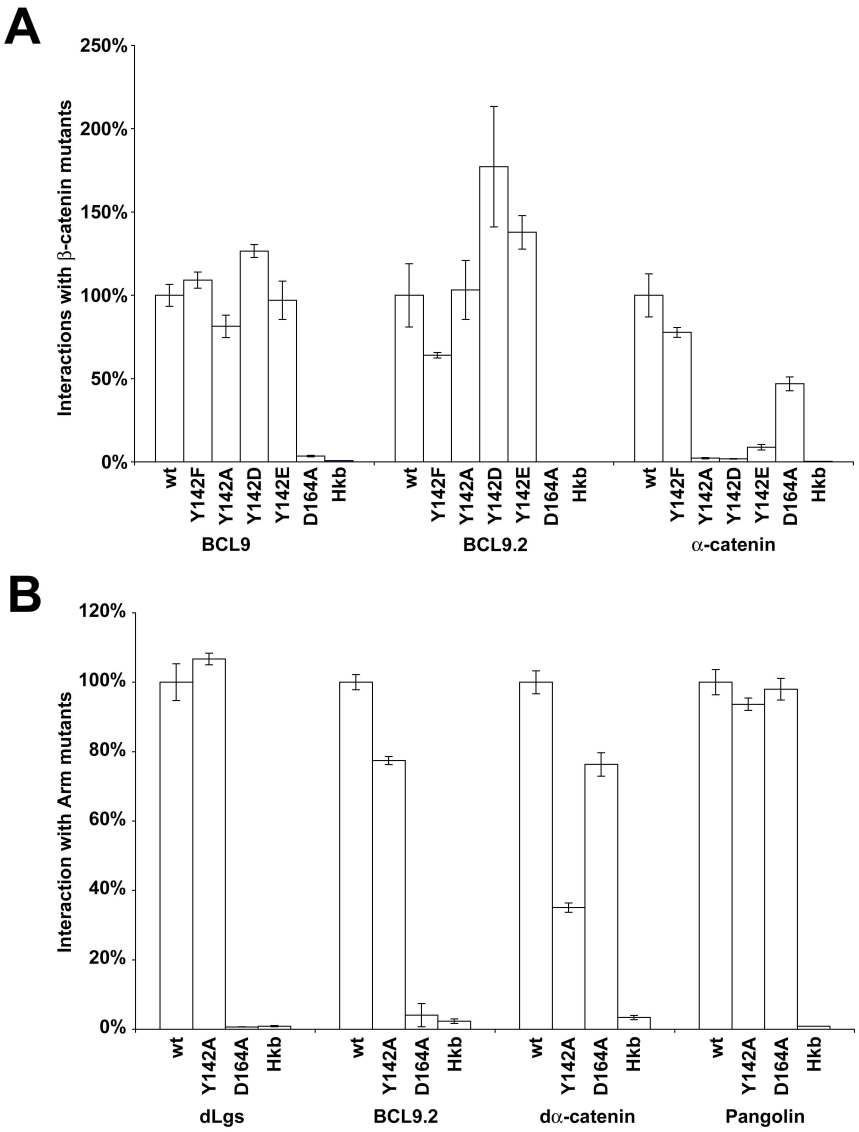

Figure 1: β-catenin/Arm-D164 is important for BCL9/Lgs and BCL9.2 binding, whereas β-catenin/Arm-Y142 is crucial for α-catenin binding.
Using a quantitative yeast two-hybrid assay the interactions of β-catenin and Armadillo mutants with BCL9, Lgs, BCL9.2, α-catenin and Pangolin (Pan) were tested. The transcription factor Huckebein (Hkb) (Bronner et al., 1994), which plays no role in Wnt/Wg signalling, served as a negative control.
(A) β-catenin mutants were tested for binding with BCL9, BCL9.2 and α-catenin. The D164A mutation abrogated binding to BCL9 and BCL9.2. The Y142 mutations do not affect binding to BCL9 or BCL9.2, suggesting that the residue itself, as well as its phosphorylation, is not necessary for BCL9 and BCL9.2 binding. However mutations of Y142 (except Y142F) do abolish α-catenin binding.
(B) Arm mutants were tested for binding with Lgs, BCL9.2, *Drosophila* α-catenin and Pan. The D164A mutation severely affects binding of Lgs and BCL9.2. The Y142A mutation however affects mainly *Drosophila* α-catenin binding and not Lgs or BCL9.2.

Constitutively active signalling of Arm^{S10} does not depend on tyrosine 142

The results from the binding and rescue experiments suggest that Y142 in Arm is necessary for adhesion but not signalling. Hence we wanted to test the Y142A mutation in a signalling assay which is not adhesion dependent. β-catenin/Arm mutants that have N-terminal truncations which delete the GSK-3β phosphorylation sites are degradation resistant and therefore overactivate the Wg signalling pathway (Pai et al., 1996). One of these constitutively active forms is Arm^{S10}, which has an N-terminal in-frame deletion but can still bind α-catenin.

Table 1. Rescue ability of *arm* transgenes

Transgene	Rescue	
None	0%	n=150
tub-Arm-wt	90%	n=190
tub-Arm-Y142A	29%	n=645
tub-Arm-D164A	0%	n=328

Females heterozygous for arm^{2a9} were crossed with males carrying different *tubulinα1*-promoter-driven rescue constructs. The percentage of rescued males containing both the arm^{2a9} allele and the *tubulinα1* rescue construct are shown. *n* indicates the male progeny that contain the *tubulinα1* rescue construct but inherited the wild-type *arm* allele from the balancer chromosome instead of the arm^{2a9} allele and therefore corresponds to the expected number of arm^{2a9} males with the *tubulinα1* rescue construct.

Ubiquitous expression in the embryo of an Arm^{S10}-wt transgene (driven by *daughterless-Gal4*) suppresses denticle formation and results in a naked cuticle (Figure 2B and (Hoffmans and Basler, 2004)). This Wg gain of function phenotype is completely abolished when the Lgs binding mutant (Arm^{S10}-D164A) is used (Figure 2D and (Hoffmans and Basler, 2004)). The Y142 mutant (Arm^{S10}-Y142A) shows a similar gain of signalling activity as Arm^{S10}-wt (Figure 2C). Thus, constitutive Arm^{S10} depends on intact D164 but not Y142 for its function. We deduce from this data that Y142 is not essential for transcriptional activity *in vivo* in *Drosophila*.

β-catenin is not dependent on tyrosine 142 for signalling *in vitro*

The experiments presented above suggest that Y142 of Arm is not necessary for Wg signalling in *Drosophila*. In order to test the effect of this mutation in β-catenin on signalling, different forms of constitutively active β-catenin (S33Y) fused to the DNA binding domain of Gal4 (G4DBD-S33Y) were compared using *UAS-luciferase* as a read out (Hoffmans et al., 2005). In Human embryonic kidney 293 (HEK293) cells, which express low levels of BCL9.2 (Adachi et al., 2004; Brembeck et al., 2004), addition of G4DBD-S33Y-wt caused strong transcriptional activation of the reporter. The D164A form, which cannot bind BCL9 and BCL9.2 is much less active. In contrast, β-catenin-Y142A is as active the wildtype form. Additionally, mimicking phosphorylation by changing Y142 to glutamic acid or aspartic acid had no consequences for activation (Figure 3A). Similar results were obtained in SW480 cells (Figure 3B) which have high levels of BCL9.2 (Adachi et al., 2004). This suggests that Y142 of β-catenin is not essential for Wnt signalling in mammalian tissue culture cells.

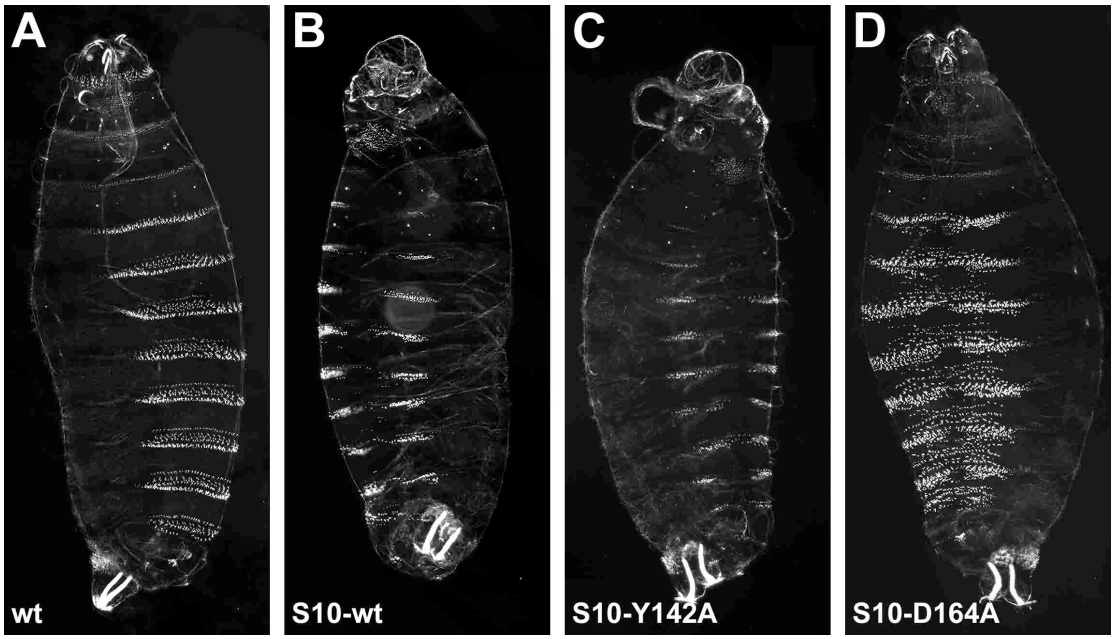

Figure 2: The Y142A mutation does not affect transcriptional activity of a constitutively active Arm
(A) The cuticle of a wildtype embryo where denticle bands alternate with naked cuticle corresponding to the alternating pattern of Wg pathway activity.
(B) Ubiquitous expression of a constitutively active Arm (Arm^{S10}) results in overactivation of the pathway, which can be seen by the ectopic naked cuticle.
(C) Ubiquitous expression of Arm^{S10} carrying the Y142A mutation leads to a similar gain of function phenotype as Arm^{S10}-wt.
(D) Ubiquitous expression of Arm^{S10} carrying the D164A mutation does not lead to a naked cuticle. It does show a slight dominant negative effect (ectopic denticles at regions that are normally naked).

Both BCL9.2 and BCL9 can functionally replace Lgs in *Drosophila* tissue culture cells and *in vivo*

It was previously shown that BCL9.2, in contrast to BCL9 and Lgs, does not depend on Pygo binding in order to function as a transcriptional activator (Adachi et al., 2004; Brembeck et al., 2004). To further explore the independence of BCL9.2 function on Pygo binding we examined the significance of the interaction *in vivo* for Wg signalling. We first addressed whether BCL9.2 can, similar to BCL9, replace Lgs in tissue culture cells and flies. In *Drosophila* S2 cells, after RNAi mediated knock-down of Lgs, signalling rescuing activity of BCL9 and BCL9.2 was measured with *wf-luciferase* (Hoffmans et al., 2005)(Figure 4). BCL9 can very effectively rescue signalling activity in the absence of Lgs and this is dependent on Pygo binding since a Pygo binding mutant BCL9 (BCL9ΔHD1, Kramps et al., 2002) cannot rescue. The BCL9.2 protein can also rescue signalling activity although not as efficiently as BCL9 (Figure 4). This is not depending on the BCL9.2 nuclear localization signal as deletion of this signal (BCL9.2Δ138-173, Brembeck et al., 2004) has no influence and is as active as full length BCL9.2. However, the ability of BCL9.2 to replace Lgs is also dependent on Pygo binding because a mutant for Pygo binding (BCL9.2ΔPyBD, Brembeck et al., 2004 and data not shown) is not able to rescue signalling activity.

BCL9 when driven by a *tubulinα1*-promoter has been shown to be able to rescue viability and limb pattern of lgs^{17E}/lgs^{21L} animals, which normally die as pharate adults

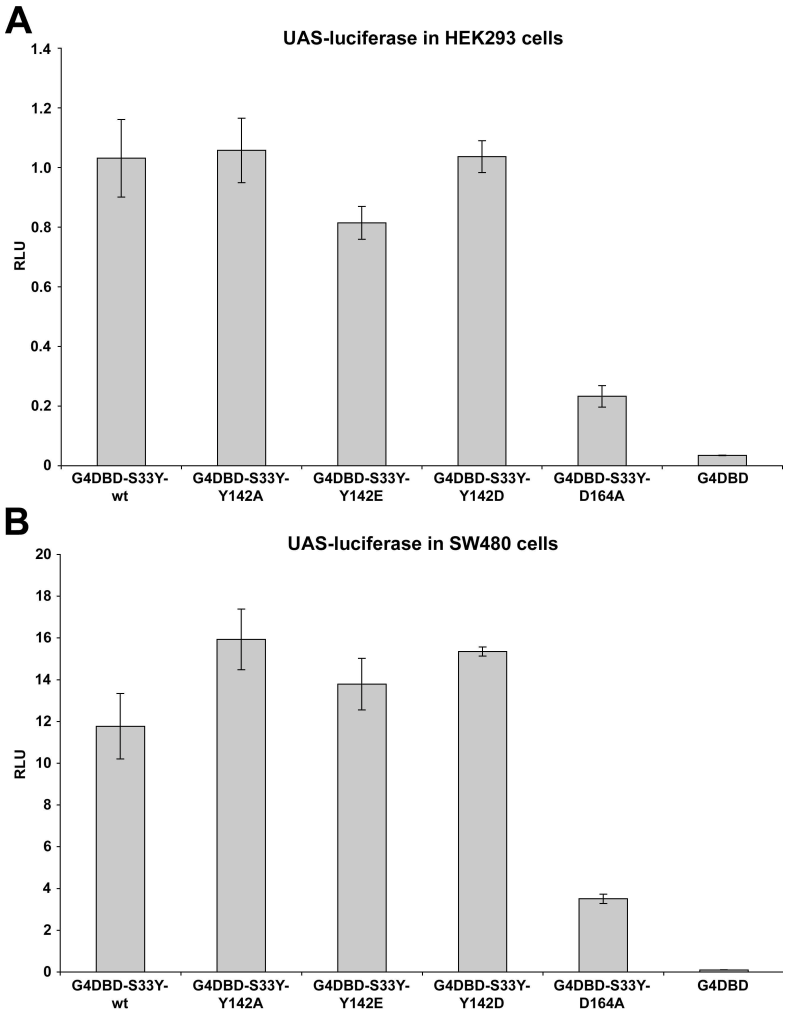

Figure 3: β-catenin-Y142 is not important for signalling in mammalian tissue culture cells
There is no difference in signalling activity between β-catenin-wt or a Y142 mutant in cells with low (HEK293) or high (SW480) BCL9.2 levels.
(A) HEK293 cells were transfected with constitutively active (S33Y) mouse β-catenin bound to the Gal4 DNA binding domain (G4DBD-S33Y). Wildtype causes a robust activation of the UAS-luciferase reporter. However, the D164A form, which is mutant for BCL9 and BCL9.2 binding, shows strongly reduced activity. The mutants for Y142 are as similarly active as wildtype.
(B) The outcome of the SW480 cell experiment is identical to (A)
Error bars represent standard deviations of triplicates. All β-catenin proteins were equally expressed as measured by western blotting (data not shown).

(Kramps et al., 2002). To explore the possibility if BCL9.2 could be a functional homolog of Lgs, *tubulinα1*-promoter driven transgenes of BCL9.2 and BCL9.2ΔPyBD were generated. BCL9.2 can rescue lgs^{17E}/lgs^{21L} animals, but this rescue is dependent on Pygo binding since BCL9.2ΔPyBD cannot rescue these animals (data not shown). These results suggest that BCL9.2, like BCL9, is a functional homologue of Lgs and that BCL9.2 needs to bind Pygo for its function.

Discussion

Members of the Wg/Wnt family are secreted signalling molecules that have a crucial role in developmental processes by regulating the transcription of their target genes. Aberrant

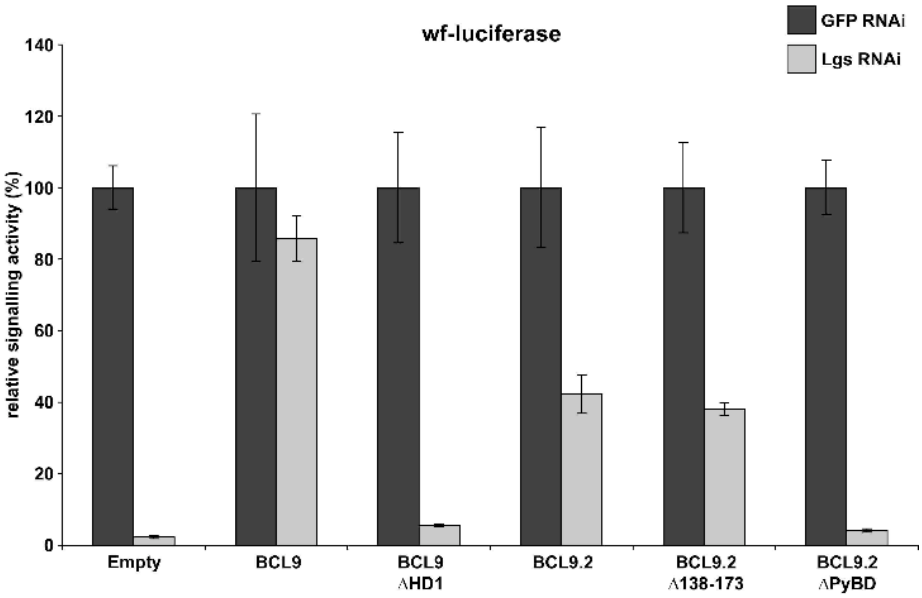

Figure 4: BCL9.2 can functionally replace lgs in S2 cells
(A) In S2 cells BCL9 can rescue Lgs RNAi as measured by the wf-luciferase reporter. This is dependent on the ability of BCL9 to bind Pygo since a binding mutant (BCL9ΔHD1) cannot rescue. BCL9.2 can partially rescue Lgs RNAi. This is not dependent on the intrinsic NLS of BCL9.2 (BCL9.2Δ138-173) but is dependent on Pygo binding (BCL9.2ΔPyBD). To activate the pathway, cells were cocultured with Wg-secreting cells (van Leeuwen et al., 1994), which resulted in a 20-fold stimulation of the wf-luciferase (data not shown). The rescue activity of the different BCL9 and BCL9.2 proteins is represented relative to the respective GFP RNAi negative control.

activation of the Wnt signalling pathway has been casually linked to human diseases and cancer (reviewed by Moon et al., 2004). Arm/β-catenin, the effector protein of the pathway, is post-transcriptionally upregulated, accumulates in the cytoplasm and enters the nucleus. There it binds to the DNA binding protein TCF/Lef and to BCL9 which recruits Pygopus to the complex. In this report we provide evidence that binding of BCL9, BCL9.2 and Lgs is not dependent on Y142 of Arm/β-catenin. Single amino acid substitutions of Y142 had no effect on Arm/β-catenin transcriptional activity both *in vivo* and in tissue culture cells. In addition we show that BCL9.2 can functionally replace Lgs in *Drosophila* S2 cells and *in vivo* and that this is dependent on Pygo binding.

It was previously reported that BCL9.2 interacted with β-catenin in a tyrosine phosphorylation dependent manner. This interaction was dependent on the presence of an active receptor tyrosine kinase. Furthermore, mutation of β-catenin-Y142 to alanine abrogated binding to BCL9.2 in this modified yeast two-hybrid system (Brembeck et al., 2004). In contrast, our binding studies show that Arm/β-catenin binding to BCL9.2 is not dependent on Y142. In agreement with our results, BCL9.2 was also identified as a β-catenin binding partner in a standard yeast two-hybrid system (Adachi et al., 2004) which lacks receptor tyrosine kinases. One explanation to account for the discrepancy between the two results may be attributed to the use of different lengths of β-catenin proteins in the binding assays. While we used full length β-catenin, the other groups used truncated versions of the

protein. Our hypothesis is that full length β-catenin more accurately reflects the *in vivo* situation.

The *arm*^{2a9} allele is the strongest *arm* allele available and has both a defect in adhesion function as well as in Wg signalling (Peifer et al., 1993; Peifer and Wieschaus, 1990). An Arm-Y142A transgene was able to rescue *arm*^{2a9} males but not as efficiently as an Arm-wt transgene. The binding studies suggest that Arm-Y142A has reduced α-catenin binding. One possible explanation for the reduced efficiency seen with Arm-Y142A could be that the adhesion defect of *arm*^{2a9} allele is not overcome completely.

BCL9.2 cannot rescue Lgs RNAi as efficiently as BCL9, this could be due to different binding affinities of BCL9 and BCL9.2 for Arm and/or Pygo. In this scenario, BCL9.2 has a lower binding affinity for Arm and/or Pygo than BCL9 and therefore the rescue by BCL9.2 would be less efficient. Consistent with this, in our yeast two-hybrid experiments we did notice that BCL9.2 bound less well to β-catenin then BCL9 did (data not shown), although it must be said that this is not a precise way of comparing binding affinities of different proteins.

We show that BCL9.2 is a functional homolog of Lgs and depends on Pygo binding for its rescue activity in flies. However, Adachi et al (2004) showed that in mammalian cells BCL9.2 is not dependent on Pygo binding for signalling but that it is dependent on the C-terminus of BCL9.2. At the amino acid level BCL9 and BCL9.2 have three highly homologues regions of 20-30 amino acids in their C-terminus (Adachi et al., 2004; Brembeck et al., 2004). Interestingly, these short clusters are missing in Lgs. For Lgs it has been shown that the only two domains important for its function are the Pygo binding domain (HD1) and the β-catenin interaction domain (HD2). A transgene containing only HD1 and HD2 fused by a triple HA tag could rescue *lgs* null mutants flies suggesting that the rest of the Lgs protein is dispensable for its function (Kramps et al., 2002). More work is needed to determine the contribution of C-terminus of BCL9 and BCL9.2 in Wnt signalling in vertebrates.

Acknowledgements:

We thank W. Birchmeier, F.H. Brembeck and R. Kemler for plasmids.

Materials and methods

Plasmid constructs

Single amino acid mutations were introduced by site-directed mutagenesis (Quickchange-Kit, Stratagene) in mouse β-catenin (amino acids 1-781) and Arm (amino acids 38-806) which were cloned in pGAD424. Human BCL9 (amino acids 199-392), mouse α-catenin (amino acids 1-750), human BCL9.2 (amino acids 232-427), Lgs (amino acids 364-555), Pan (amino acids 1-129), *Drosophila* α-catenin (amino acids 1-394) were cloned into pBTM116 (Bartel and Fields, 1997). All constructs were verified by sequencing.

pcDNA3.1 (Invitrogen) or pPacPL (Koelle and Hogness, 1992) vectors were used for expression of cDNAs in mammalian cells or S2 cells, respectively.

For simplicity, we use the amino acid numbering of β-catenin for both β-catenin and Arm throughout the text. β-catenin Y142 and D164 would correspond to Arm Y150 and D172.

dsRNA production

Templates for GFP and Lgs dsRNA synthesis were generated by PCR using the following primers containing 5' T7 promoter tails:

fGFP 5'T7CTTTTCACTGGAGTTGTCC, rGFP 5'T7ATCCATGCCATGTGTAATCC, product length: 0.68kb;

fLgs 5'T7GGCATGCGTCCACATGCC, rLgs 5'T7ATTTGTTGACAAAGAACGTTG, product length: 0.58kb;

dsRNA was then synthesized from these templates using the Ambion Megascript kit. For RNAi in S2 cells (Clemens et al., 2000) 1μg of dsRNA was used per well (96 well plate).

Cell culture

S2 cell transfections were performed in 96 well plates using polyethylenimine (PEI, Polysciences, Inc) with a total of 500 ng of DNA per 3 wells (50 ng *wf-luc*, 100 ng *actin5c-renilla*, 50 ng *tubulinα1-dfrizzled2*, 50 ng rescue constructs and 250 ng empty vector). The Wg signalling pathway was induced 48 hr after transfection by adding heat-shocked S2 cells stably transfected with Wg cDNA under the control of a heat-shock promoter (van Leeuwen et al., 1994).

HEK293 cell transfections were performed in 24 well plates using Lipofectamine (Invitrogen) with a total of 0.8 ug of DNA per 3 wells (300 ng *UAS-luc*, 100 ng pCMV-RL (Promega), 300 ng Gal4DBD constructs, 100 ng of empty vector).

HeLa cell transfections were performed in 24 well plates using Lipofectamine and PLUS reagent (Invitrogen) with a total of 1.2 ug of DNA per 3 wells (400 ng *UAS-luc*, 100 ng pCMV-RL (Promega), 600 ng G4DBD constructs, 100 ng of empty vector).

Luciferase activities were determined after 72 hr for S2 cells and after 48 hr for HEK293 and HeLa cells using the Dual-Luciferase Assay System (Promega).

Yeast two-hybrid

The yeast two-hybrid system was used as described in chapter 3 of Bartel and Fields (1997). Interactions between proteins were measured using the quantitative 'Liquid Culture Assay Using ONPG as Substrate' (Clontech, Yeast Protocols Handbook, http://www.clontech.com/clontech/Techinfo/manuals).

Transgenes

For embryonic experiments *arm* transgenes were expressed from UAS-constructs under control of the *daughterless-Gal4* driver (Wodarz et al., 1995). Several independent lines of arm^{S10}*-wt*, arm^{S10}*-Y142A* and arm^{S10}*-D164A* transgenes were established and tested. For rescue experiments full-length *arm* transgenes were driven by the *tubulinα1* (*tub*) promoter (Basler and Struhl, 1994). All *arm* coding regions used contain a c-Myc epitope in their C-terminal region at the same position as the original arm^{S10} (Pai et al., 1997).

References

Aberle, H., Bauer, A., Stappert, J., Kispert, A. and Kemler, R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. *Embo J* **16**, 3797-804.

Aberle, H., Schwartz, H., Hoschuetzky, H. and Kemler, R. (1996). Single amino acid substitutions in proteins of the armadillo gene family abolish their binding to alpha-catenin. *J Biol Chem* **271**, 1520-6.

Adachi, S., Jigami, T., Yasui, T., Nakano, T., Ohwada, S., Omori, Y., Sugano, S., Ohkawara, B., Shibuya, H., Nakamura, T. et al. (2004). Role of a BCL9-related beta-catenin-binding protein, B9L, in tumorigenesis induced by aberrant activation of Wnt signaling. *Cancer Res* **64**, 8496-501.

Bartel, P. L. and Fields, S. (1997). The Yeast Two-Hybrid System. New York, NY: Oxford University Press.

Basler, K. and Struhl, G. (1994). Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein. *Nature* **368**, 208-14.

Behrens, J., Jerchow, B. A., Wurtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kuhl, M., Wedlich, D. and Birchmeier, W. (1998). Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. *Science* **280**, 596-9.

Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R. and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* **382**, 638-42.

Belenkaya, T. Y., Han, C., Standley, H. J., Lin, X., Houston, D. W. and Heasman, J. (2002). pygopus Encodes a nuclear protein essential for wingless/Wnt signaling. *Development* **129**, 4089-101.

Brembeck, F. H., Schwarz-Romond, T., Bakkers, J., Wilhelm, S., Hammerschmidt, M. and Birchmeier, W. (2004). Essential role of BCL9-2 in the switch between beta-catenin's adhesive and transcriptional functions. *Genes Dev* **18**, 2225-30.

Bronner, G., Chu-LaGraff, Q., Doe, C. Q., Cohen, B., Weigel, D., Taubert, H. and Jackle, H. (1994). Sp1/egr-like zinc-finger protein required for endoderm specification and germ-layer formation in Drosophila. *Nature* **369**, 664-8.

Brunner, E., Peter, O., Schweizer, L. and Basler, K. (1997). pangolin encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in Drosophila. *Nature* **385**, 829-33.

Clemens, J. C., Worby, C. A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B. A. and Dixon, J. E. (2000). Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. *Proc Natl Acad Sci U S A* **97**, 6499-503.

Drees, F., Pokutta, S., Yamada, S., Nelson, W. J. and Weis, W. I. (2005). alpha-Catenin Is a Molecular Switch that Binds E-Cadherin-beta-Catenin and Regulates Actin-Filament Assembly. *Cell* **123**, 903-15.

Hecht, A., Vleminckx, K., Stemmler, M. P., van Roy, F. and Kemler, R. (2000). The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. *Embo J* **19**, 1839-50.

Hoffmans, R. and Basler, K. (2004). Identification and in vivo role of the Armadillo-Legless interaction. *Development* **131**, 4393-400.

Hoffmans, R., Stadeli, R. and Basler, K. (2005). Pygopus and legless provide essential transcriptional coactivator functions to armadillo/beta-catenin. *Curr Biol* **15**, 1207-11.

Ikeda, S., Kishida, S., Yamamoto, H., Murai, H., Koyama, S. and Kikuchi, A. (1998). Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin. *Embo J* **17**, 1371-84.

Kishida, S., Yamamoto, H., Ikeda, S., Kishida, M., Sakamoto, I., Koyama, S. and Kikuchi, A. (1998). Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of beta-catenin. *J Biol Chem* **273**, 10823-6.

Koelle, M. and Hogness, D. (1992). Technical Notes: Plasmid vectors for expressing proteins in tissue culture cells or study enhancer function. *Drosophila Information Newsletter*, 401-415.

Kramps, T., Peter, O., Brunner, E., Nellen, D., Froesch, B., Chatterjee, S., Murone, M., Zullig, S. and Basler, K. (2002). Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. *Cell* **109**, 47-60.

Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G. H., Tan, Y., Zhang, Z., Lin, X. and He, X. (2002). Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* **108**, 837-47.

Moon, R. T., Kohn, A. D., De Ferrari, G. V. and Kaykas, A. (2004). WNT and beta-catenin signalling: diseases and therapies. *Nat Rev Genet* **5**, 691-701.

Pai, L. M., Kirkpatrick, C., Blanton, J., Oda, H., Takeichi, M. and Peifer, M. (1996). Drosophila alpha-catenin and E-cadherin bind to distinct regions of Drosophila Armadillo. *J Biol Chem* **271**, 32411-20.

Pai, L. M., Orsulic, S., Bejsovec, A. and Peifer, M. (1997). Negative regulation of Armadillo, a Wingless effector in Drosophila. *Development* **124**, 2255-66.

Parker, D. S., Jemison, J. and Cadigan, K. M. (2002). Pygopus, a nuclear PHD-finger protein required for Wingless signaling in Drosophila. *Development* **129**, 2565-76.

Peifer, M., Orsulic, S., Sweeton, D. and Wieschaus, E. (1993). A role for the Drosophila segment polarity gene armadillo in cell adhesion and cytoskeletal integrity during oogenesis. *Development* **118**, 1191-207.

Peifer, M. and Wieschaus, E. (1990). The segment polarity gene armadillo encodes a functionally modular protein that is the Drosophila homolog of human plakoglobin. *Cell* **63**, 1167-76.

Pokutta, S. and Weis, W. I. (2000). Structure of the dimerization and beta-catenin-binding region of alpha-catenin. *Mol Cell* **5**, 533-43.

Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S. C., Grosschedl, R. and Bienz, M. (1997). LEF-1, a nuclear factor coordinating signaling inputs from wingless and decapentaplegic. *Cell* **88**, 777-87.

Stadeli, R. and Basler, K. (2005). Dissecting nuclear Wingless signalling: Recruitment of the transcriptional co-activator Pygopus by a chain of adaptor proteins. *Mech Dev* **122**, 1171-82.

Takemaru, K. I. and Moon, R. T. (2000). The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression. *J Cell Biol* **149**, 249-54.

Thompson, B., Townsley, F., Rosin-Arbesfeld, R., Musisi, H. and Bienz, M. (2002). A new nuclear component of the Wnt signalling pathway. *Nat Cell Biol* **4**, 367-73.

Thompson, B. J. (2004). A complex of Armadillo, Legless, and Pygopus coactivates dTCF to activate wingless target genes. *Curr Biol* **14**, 458-66.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A. et al. (1997). Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell* **88**, 789-99.

van Leeuwen, F., Samos, C. H. and Nusse, R. (1994). Biological activity of soluble wingless protein in cultured Drosophila imaginal disc cells. *Nature* **368**, 342-4.

Yamada, S., Pokutta, S., Drees, F., Weis, W. I. and Nelson, W. J. (2005). Deconstructing the cadherin-catenin-actin complex. *Cell* **123**, 889-901.

2.4 *pygo* double knock-out mouse embryonic fibroblasts

In order to elucidate the function of Lgs and Pygo in the Wnt pathway in a vertebrate model organism, knock-out (KO) mice were generated by members of the lab of Michel Aguet (ISREC, Lausanne). Mice have two paralogs of Pygo (mPygo1 and mPygo2) and the KO constructs were designed in such a way that in both cases the NHD as well as the PHD of Pygo was deleted. The direct KO of mPygo1 is viable and fertile and does not show any phenotypes (after 6 months). In contrast, the direct KO of mPygo2 is lethal at E14,5. The phenotype of the double KO of both alleles is indistinguishable from mPygo2 KO (personal communication, Aguet lab members).

We received from the Aguet lab *pygo1*; *pygo2* mouse embryonic fibroblasts (MEF), generated from E13,5 embryos. A western blot showed that Pygo double KO MEFs indeed do not have any Pygo protein (Figure III). In the same experiment the Pygo levels between 293T (human embryonic kidney), NIH3T3 (a widely used MEF cell line) and wildtype MEFs (also received from the Aguet lab) were compared. These three cell lines seem to express equal amounts of Pygo protein (Figure III).

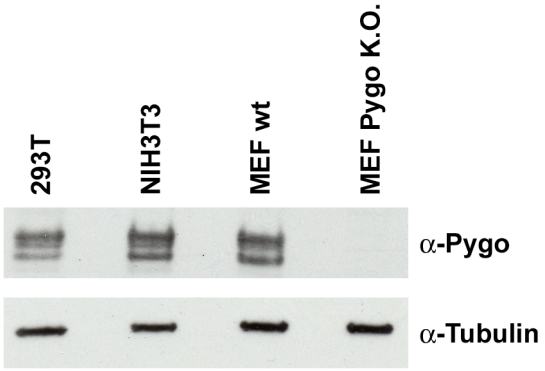

Figure III: *pygo* KO MEFs do not express Pygo protein.
Western blot on lysates show that pygo1 -/- ; pygo2 -/- MEFs do not express Pygo. Whereas, 293T, NIH3T3 and wildtype (wt) MEF cells express equal amounts of Pygo. For this western blot the hPygo2b antibody was used, which is a rabbit polyclonal antibody directed against amino acid 34-189 (contains the NHD).

We wanted to know if Pygo has the same function in Wnt signalling in mammals as it has in *Drosophila*. Therefore we tested if the *pygo* double knock-out cells are impaired in Wnt signalling. The results presented below on the double KO MEFs are preliminary and should not be considered conclusive, but they give a starting point for further experiments. A comparison was made between the signalling capacity of β-catenin in NIH3T3, wt MEF and *pygo* KO MEF cell lines. For this we made use of an *UAS-luciferase* reporter (Hoffmans et al., 2005) and a constitutively active β-catenin (ΔN, lacking the first 129 amino acids) fused to the DNA binding domain of Gal4 (G4DBD-ΔN). In the wt MEFs and in the NIH3T3 cells G4DBD-ΔN-wt can strongly activate the reporter (Figure IVA and B). Adding additional Pygo has a minimal effect on the already robust activated reporter in wt MEFs and NIH3T3 cells. In the

pygo KO MEF cell line, DNA tethered β-catenin (G4DBD-ΔN-wt) led to transcriptional activation of the reporter in the absence of Pygo (Figure IVC). When Pygo was added, this resulted in a stronger activation of the reporter. In these signalling assays the KO MEFs without addition of Pygo show lower signalling activity then wt MEFs and NIH3T3 (compare fold induction of the G4DBD to G4DBD-ΔN-wt in all three cell lines). When Pygo is added to the KO MEFs, these cells show a similar signalling activity as wt MEFs and NIH3T3 cells do.

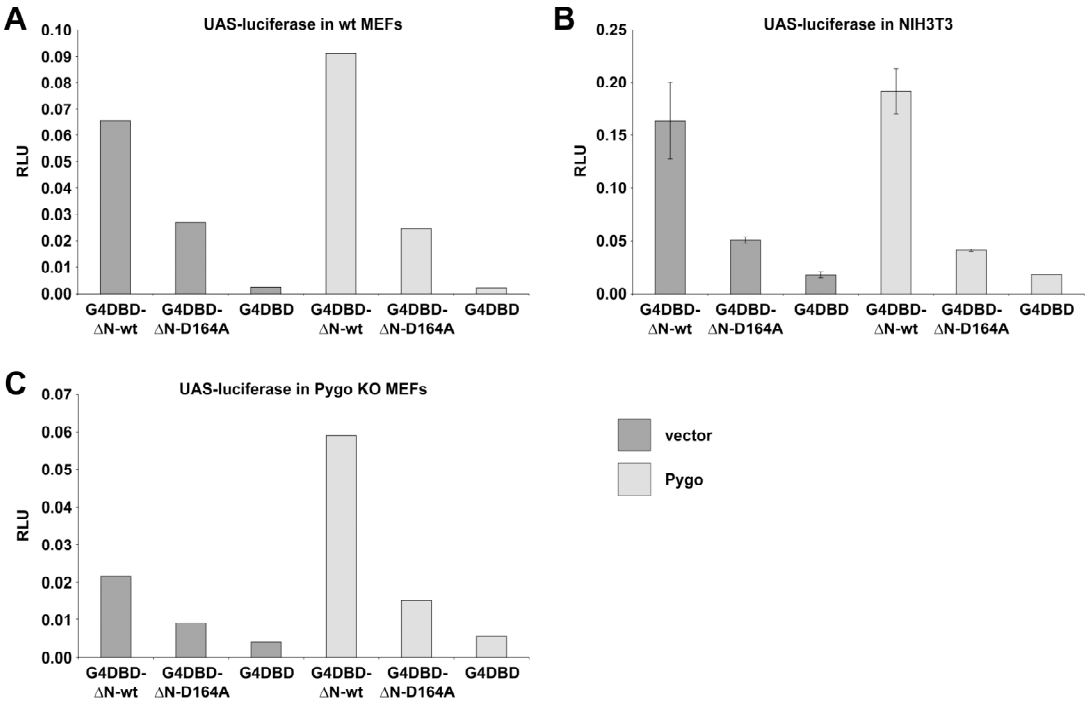

Figure IV: Pygopus enhances transactivation of nuclear β-catenin complex
Evaluation of β-catenin transactivation capacity in *pygo* KO, wildtype (wt) and NIH3T3 MEF cell lines. Relative reporter-gene activities induced by two different versions of DNA-tethered β-catenin (G4DBD-ΔN-wt and D164A) were assayed after 48 hours.
(A) In wt MEFs, G4DBD-ΔN-wt can strongly activate the reporter. G4DBD-ΔN-D164A is less active. The addition of extra Pygo to G4DBD-ΔN-wt or G4DBD-ΔN-D164A only has a minor effect on reporter activity. Wt MEFs were transfected in 10 cm plates with 7 µg of DNA (1 ug *UAS-luciferase*, 1 ug *pCMV-RL* (Promega), 1 ug eGFP, 2 ug empty vector or 2 ug hPygo2 and 2 ug of either *G4DBD* constructs) using the nucleofector technology (Amaxa).
(B) NIH3T3 MEFs behave the same as wt MEFs. NIH3T3 MEFs were transfected in 24-well plates with 0.7 µg of DNA per 2 wells (100 ng *UAS-luciferase*, 100 ng *pCMV-RL* (Promega), 100 ng eGFP, 200 ng empty vector or 200 ng hPygo2 and 200 ng of either *G4DBD* constructs) using the PLUS reagent and lipofectamine (Invitrogen). Error bars represent standard deviations of duplicates.
(C) In *pygo* KO MEFs, G4DBD-ΔN-wt can activate the reporter. Whereas G4DBD-ΔN-D164A is less active. Adding both Pygo and G4DBD-ΔN-wt results in enhanced activity, which is comparable to the activity of G4DBD-ΔN-wt in wt MEFs. In this particular experiment adding extra Pygo also boosted the signal activity of G4DBD-ΔN-D164A, but this not observed consistently. *pygo* KO MEFs were transfected in 10 cm plates with 7 µg of DNA (1 ug *UAS-luciferase*, 1 ug *pCMV-RL* (Promega), 1 ug eGFP, 2 ug empty vector or 2 ug hPygo2 and 2 ug of either *G4DBD* constructs) using Amaxa nucleofector technology).

In a *pygo* null background one would expect that G4DBD-ΔN-wt and G4DBD-ΔN-D164A would show exactly the same level of signalling. Interestingly in the *pygo* KO MEF cell line G4DBD-ΔN-D164A does not behave exactly the same as G4DBD-ΔN-wt does (Figure IVA). The protein that no longer can bind BCL9 and BCL9.2 has a further reduced signalling capacity compared to the protein that still can bind BCL9 and BCL9.2. This might suggest that BCL9 and/or 9.2 have a function in nuclear Wnt signalling surpassing the recruitment of Pygo to β-catenin. One possible explanation could be that BCL9 and/or 9.2 interfere with binding of

an inhibitor to β-catenin signalling. In the case of G4DBD-ΔN-wt this inhibitor cannot bind because of BCL9/9.2 binding. However, in the case of G4DBD-ΔN-D164A which cannot bind BCL9/9.2, the inhibitor is not prevented from binding resulting in a reduction in signalling. Another possibility could be that BCL9.2 has transactivation activity independent of Pygo as was suggested by Adachi et al. (2004). The extra signalling of G4DBD-ΔN-wt compared to G4DBD-ΔN-D164A in a *pygo* null background would then be caused by the transactivation activity of BCL9.2. It will be interesting to see if there is a difference in transcriptional activity between these two β-catenin versions in a *BCL9*; *BCL9.2* double knock-out cells.

In *Drosophila* S2 cells and in flies it was shown that a Pygo protein with a mutation in the NHD domain (F78A) is not functional in signalling assays (Hoffmans et al., 2005; Stadeli and Basler, 2005). We wanted to test in a mammalian system if disruption of the NHD is important for Pygo function. The *pygo* KO MEF cell line provides an excellent opportunity to examine this. In *pygo* KO MEFs addition of Pygo leads to an increase in reporter activity (Figure IVA). However addition of Pygo-F78A or Pygo lacking amino acids 74-78 resulted in a similar increase. Also deletion of amino acids 70-81 had only a minor effect on signalling activity (data not shown, Figure V). This is clearly different from the situation in *Drosophila* where an intact NHD is essential for Pygo function. It could be that in *Drosophila* an intact NHD is more important then in mammals. It will be interesting to see if a Pygo construct that only contains the PHD domain is as active as wt Pygo in the KO cells.

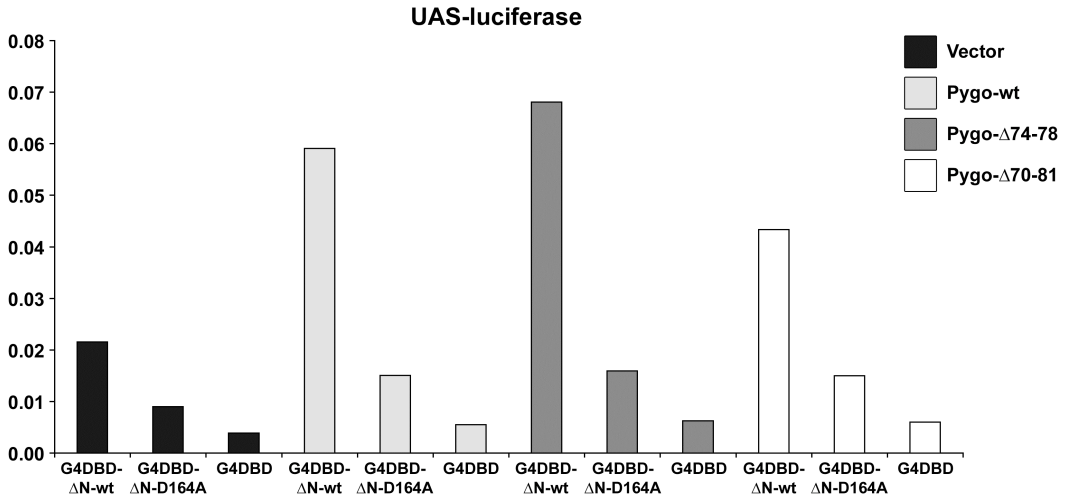

Figure V: Intact NHD domain of Pygo not important for transactivation in MEFs

Evaluation of different forms of Pygo for transactivation capacity in *pygo* KO MEF cell line. Relative reporter-gene activities were measured. G4DBD-ΔN-wt induced the *UAS-Luciferase* reporter, this activity can be enhanced by expression of Pygo-wt but also by Pygo-Δ74-78 and Pygo-Δ70-81.

2.5 Signalling outputs of the N- and C-terminal activating arms of Arm/β-catenin

The Arm/β-catenin protein contains three domains that are important for Wnt signalling (Orsulic and Peifer, 1996). Arm repeats 1-4 are necessary and sufficient for binding to Lgs (Hoffmans and Basler, 2004; Kramps et al., 2002; Stadeli and Basler, 2005), whereas Arm repeats 3-8 are used by the DNA binding protein Pangolin/TCF/LEF (Behrens et al., 1996; Brunner et al., 1997; Graham et al., 2000; van de Wetering et al., 1997). Finally, Arm repeats 11-12 together with the C-terminal domain (Tutter et al., 2001) are necessary for the binding of a multitude of proteins that are involved in the transcription process, namely CBP/p300, Parafibromin and Brg-1 (Barker et al., 2001; Hecht et al., 2000; Takemaru and Moon, 2000; Mosimann et al submitted). We wanted to evaluate what the contributions are of Lgs binding (Lgs branch) versus binding of C-terminal partners (C-terminal branch) to Arm/β-catenin with respect to signalling in both *Drosophila* and mammals.

Arm/β-catenin with a single amino acid mutation (D164A) in Arm repeat 1 is severely impaired in Lgs binding and in signalling. However, it was shown that Arm-D164A is functional at the adherens junctions (Hoffmans and Basler, 2004). In *Drosophila* there are several *arm* mutants that have a premature stop which results in a (partial) deletion of the C-terminus (ΔC). These mutants have severe Wnt signalling defects but they have no adhesion defects (Peifer et al., 1993; Peifer and Wieschaus, 1990). These *arm-ΔC* mutants are most likely compromised in binding of CBP/p300 and Parafibromin (Takemaru and Moon, 2000; Mosimann et al., submitted).

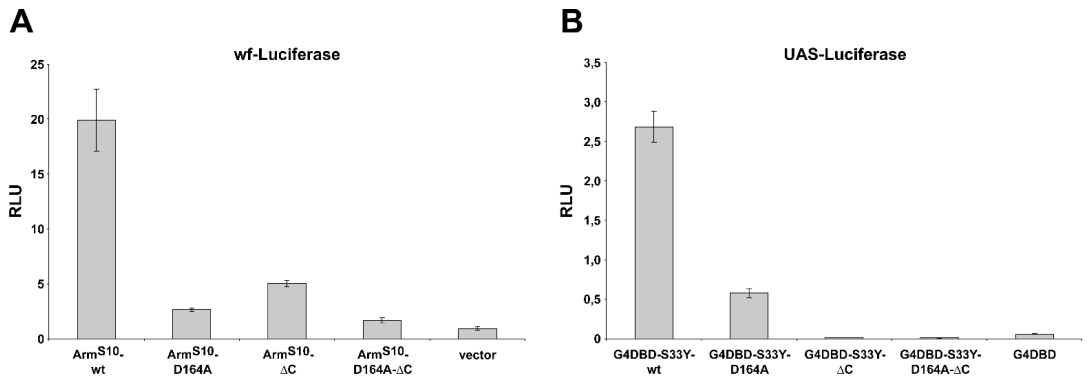

Figure VI: Evaluation of Lgs branch versus C-terminal branch of Arm/β-catenin in signalling.
(A) *Drosophila* clone 8 cells were transfected in 24 well-plates with 4 µg DNA per 3 wells (100 ng *wf-luciferase*, 100 ng *actin5c-renilla*, 100 ng *tubulinα1-Gal4*, 200 ng of *UAS-Arm^{S10}* or vector). Arm^{S10}-wt can strongly activate the reporter (measured after 48 hours) and for this Lgs binding is necessary (Arm^{S10}-D164A). There is a lesser dependence on an intact C-terminus (Arm^{S10}-ΔC). The double mutant is almost signalling dead (Arm^{S10}-D164A-ΔC).
(B) Human embryonic kidney 293 cells were transfected in 24-well plates with 0.8 µg of DNA per 3 wells (200 ng *UAS-luciferase*, 100 ng *pCMV-RL* (Promega), 300 ng empty vector and 200 ng of either *G4DBD* constructs). Constitutively active (S33Y) mouse β-catenin bound to the Gal4 DNA binding domain (G4DBD-S33Y-wt) causes a robust activation of the UAS-luciferase reporter as measured after 48 hours. However, the mutant for BCL9 binding (G4DBD-β-catenin-D164A) shows strongly reduced activity. The C-terminal deleted versions (G4DBD-S33Y-ΔC and G4DBD-S33Y-D164A-ΔC) are completely signalling incompetent. Expression levels among G4DBD-S33Y constructs were equal as measured by western blotting (data not shown).
Error bars represent standard deviations of triplicates.

The signalling capacity of different forms of constitutively active Arm (Arm^{S10}) were compared in *Drosophila* clone 8 cells. Arm^{S10}-wt is a very potent activator of the *wf-luciferase* reporter (Hoffmans et al., 2005). An Arm^{S10} version that can no longer bind Lgs (Arm^{S10}-D164A) cannot significantly activate the reporter, whereas an Arm^{S10} that is lacking the C-terminus (Arm^{S10}-ΔC) still retains some signalling activity. A combination of the two mutants (Arm^{S10}-D164A-ΔC) is completely signalling dead (Figure VIA). In agreement with this is the NLS-Arm experiment in *Drosophila* S2 cells (See Figure II), which showed that NLS-ArmΔC was more active then NLS-Arm-D164A and that NLS-Arm-D164A-ΔC is not active at all. This suggests that in *Drosophila* cells the C-terminus contributes less then Lgs binding to signalling.

In order to compare different forms of constitutively active β-catenin (S33Y) in HEK293 cells the *UAS-luciferase* reporter (Hoffmans et al., 2005) was used. β-catenin-S33Y was fused to the DNA binding domain of Gal4 (G4DBD-S33Y-wt) and caused strong transcriptional activation of the reporter (Figure VIB). The Lgs binding mutant is not a potent activator (G4DBD-S33Y-D164A) and the C-terminal domain deletion mutants (G4DBD-S33Y-ΔC and G4DBD-S33Y-D164A-ΔC) cannot activate the reporter at all. Thus in mammalian cells the C-terminus is more important for signalling then Lgs binding.

These results hint at a difference between mammals and *Drosophila* regarding the contributions of the Lgs branch versus the C-terminal branch in Arm/β-catenin signalling. In *Drosophila* cells the Lgs branch seems to be more important for signalling since the D164A mutant is less active then the ΔC mutant. However, in mammalian tissue culture, the C-terminal branch seems more important.

Table I. Rescue ability of *arm* transgenes

Transgene	*Rescue (%)*	*n*
None	0	150
Tub-Arm-wt	96	166
Tub-Arm-D164A	0	328
Tub-Arm-ΔC	4	356
Tub-Arm-D164A-ΔC	0	55

Females heterozygous for arm^{2a9} were crossed with males carrying different *tubulinα1*-promoter-driven rescue constructs. The percentage of rescued males containing both the arm^{2a9} allele and the *tubulinα1* rescue construct are shown. *n* indicates the male progeny that contain the *tubulinα1* rescue construct but inherited the wild-type *arm* allele from the balancer chromosome instead of the arm^{2a9} allele and therefore corresponds to the expected number of arm^{2a9} males with the *tubulinα1* rescue construct.

To investigate the relative inputs of the Lgs branch and the C-terminal branch of Arm in the transcriptional activity in *Drosophila in vivo* the ability of different transgenes to rescue *arm* null mutant animals (arm^{2a9}) was analysed. Hemizygous arm^{2a9} males die as embryos. With a *tubulinα1*-promoter-driven Arm-wt transgene these *arm* mutants can be rescued to

adulthood without any obvious phenotypes. In contrast, *arm*^{2a9} males die as embryos or early larvea when these Arm transgenes contain the D164A mutation or the combination of D164A-ΔC. Surprisingly, an Arm-ΔC transgene also has some rescue activity, it can rescue hemizygous *arm*^{2a9} males to adulthood without any obvious phenotypes but with a very low efficiency (Table I). This suggests that *in vivo* in *Drosophila* the Lgs branch is more important than the C-terminal branch for Wnt signalling. These results are consistent with the *Drosophila* clone 8 cell experiments where the Lgs branch appeared to be more important than the C-terminal branch.

To elucidate the functional importance of the two branches in a vertebrate model organism, we started with the generation of embryonic stem (ES) cells where the endogenous β-catenin protein (Figure VIIA) is replaced by a mutant one. Three different knock-in lines will be created, containing either β-catenin-D164A, β-catenin-ΔC or the combined mutant β-

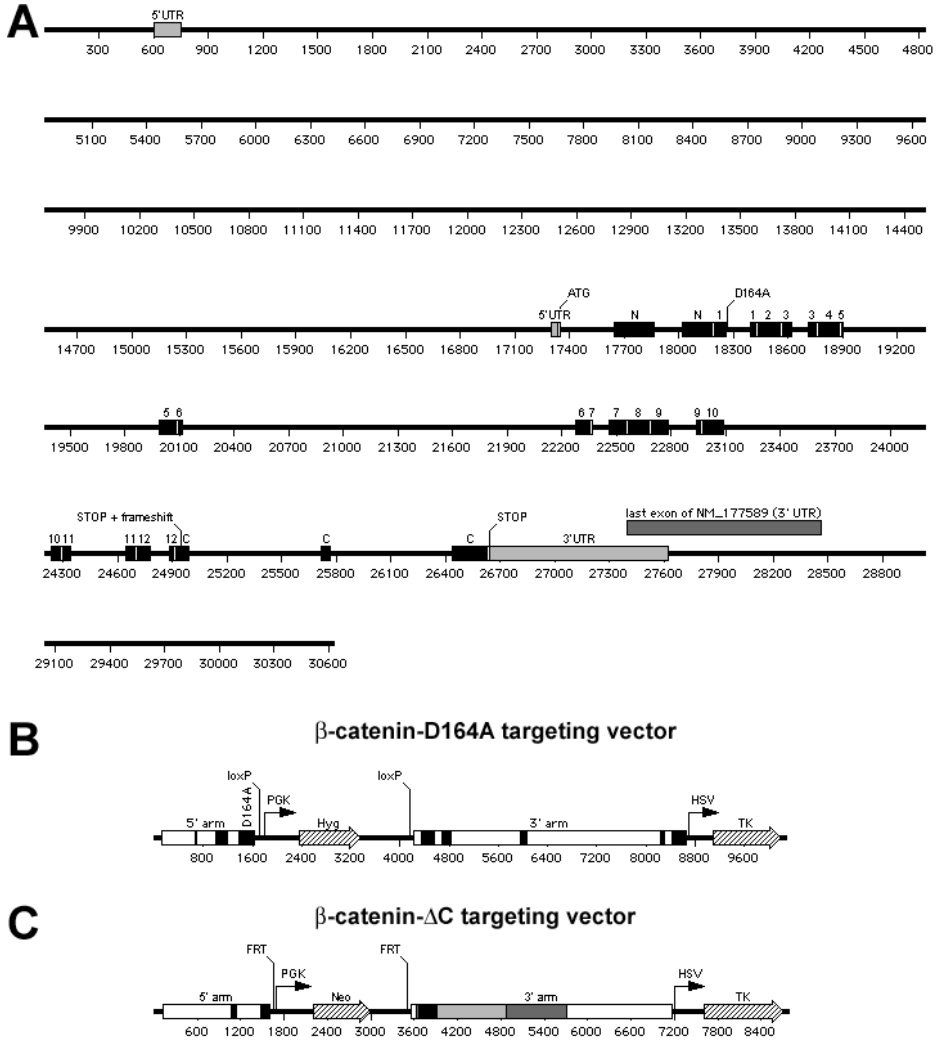

Figure VII: β-catenin genomic region and the targeting vectors
(A) The genomic region of mouse β-catenin. The exon are shown as blocks, the Arm repeats are indicated as numbers (1-12) and N/C stands for the respective terminus.
(B) The β-catenin-D164A targeting vector. The 5' arm contains exon 2-4 and the 3' arm exon 5-8 and part of 9. In exon 4 the amino acid D164 is changed to alanine.
(C) The β-catenin-ΔC targeting vector. The 5' arm contains exon 11-12 and the 3' arm exon 13 which is fused to exon 15 (exon 14 is deleted). In exon 13 amino acid 673 is changed to a stop codon and is followed by a frame shift.

catenin-D164A-ΔC. These three different ES cell lines will be injected in blastocysts and mice will be generated.

Two different targeting vectors were cloned both containing a 5' and 3' arm for homologuos recombination and integration into the genomic DNA of ES cells. The β-catenin-D164A vector (Figure VIIB) has a 5' arm where amino acid D164 is mutated to an alanine (exon 4). This targeting vector also has a PGK-promoter-driven Hygromycin cassette flanked by loxP sites for positive selection and a HSV-promoter-driven Thymidine Kinase cassette for negative selection. The β-catenin-ΔC (Figure VIIC) vector has a 3' arm where part of exon 13 was fused to exon 15 creating a stop codon after amino acid 672 followed by a frame shift. In case of read through, the frame shift will ensure a deletion of the C-terminus. Due to the fusion of the exons, the stop codon is in the last exon of the gene and thus nonsense mediated mRNA decay should not take place. In addition, this targeting vector has a PGK-promoter-driven Neomycin cassette flanked by FRT sites for positive selection and a HSV-promoter-driven Thymidine Kinase cassette for negative selection.

3. Appendix

3.1 Constructs

Abbreviations:
aa amino acid
d *Drosophila* protein
h human protein
m mouse protein
m/h hybrid between mouse and human proteins

pRH1 pBTM116.2-BCL9 (aa 199-392)
pKX-BCL9 NcoI x XhoI ligated into pBTM116.2 NcoI x XhoI

pRH2 pBS-mβ-cat (aa 129-421)

pRH3 pGAD424-hPygo2
pBTM116.2-hPygo2 EcoRI ligated into pGAD424 EcoRI

pRH5 pJP156-BCL9N (aa 1-732)
pGT-BCL9N NotI B. x XhoI ligated into pJP156 NcoI B. x SalI

pRH7 pGAD424-BCL9N (aa 1-732)
pGT-BCL9N NotI B. x XhoI ligated into pGAD424 BamHI B. x SalI

pRH8 pBTM116-m/hβ-cat (aa 129-781) **NotI not in frame**
pGAD424-m/hβ-cat (aa 129-781) BamHI x SalI ligated into pBTM116 BamHI x SalI

pRH9 pBS-m/hβ-cat (aa 129-781) **NotI not in frame**
pGAD424-m/hβ-cat (aa 129-781) SmaI x SalI ligated into pBS SmaI x SalI

pRH10 pK378-m/hβ-cat (aa 129-781) **NotI not in frame**
pRH9 XbaI x SalI ligated into pK378 XbaI x SalI

pRH11 pK378-m/hβ-cat (aa 129-781) **NotI in frame**
pGAD424-m/hβ-cat (aa 129-781) BamHI B. x SalI ligated into pK378 BamHI B. x XhoI

pRH14 pGAD424.1-hTcf4 (aa 1-130) **contain Q27R**
pET-hTcf4 (aa 1-130) BamHI x XhoI ligated into pGAD424.1 BamHI x SalI

pRH15 pBTM116RI-hTcf4 (aa 1-130) **contain Q27R**
pRH14 EcoRI x PstI ligated into pBTM116 EcoRI x PstI

pRH17 pBTM116RI-m/hβ-cat (aa 129-781)
pGAD424.1-m/hβ-cat (aa 129-781) BamHI x SalI B. ligated into pRH14 BamHI x PstI B.

pRH18 pJP156-m/hβ-cat (aa 129-781)
pRH9 XbaI x XhoI ligated into pJP156 XbaI x SalI

pRH19 pBTM116-α-cat **Not OK**
p29 XbaI B. x EcoRV ligated into pBTM116 SmaI

pRH21 pBTM116.2-Lgs (HD2)
Lgs? NcoI x NotI ligated into pBTM116.2 NcoI x NotI

pRH22 pGAD424-Hkb
Hkb? EcoRI x XhoI ligated into pAGD424 EcoRI x SalI

pRH23 pBTM116RI-m/hβ-cat ΔC (aa 129-662)
pRH24 BamHI x BglII B. ligated into pRH15 BamHI x PstI B.

pRH24 pGAD424.1-m/hβ-cat WT-R ΔC (aa 129-662)
pGAD424.1-ß-cat WT-R (aa 129-781) NdeI B. x SalI B. religated

pRH25 pK378-m/hβ-cat ΔC (aa 129-662)
pRH11 ApaI B. x NdeI B. religated (129-662)

pRH26 pBS-m/hβ-cat ΔC (aa 129-662)
pRH9 NdeI B. x Sal I B. religated

pRH27 pBTM116-BCL9N
pRH7 SmaI x BglII ligated into pBTM116 SmaI x BamHI

pRH28 pJP156-m/hβ-cat ΔC (aa 129-662)
pRH26 XbaI x XhoI ligated into pJP156 XbaI x SalI

pRH29 pBridgeMII-hTcf4 (aa 1-130)-ß-cat (aa 129-662) **not yet sequenced**
pRH25 NotI ligated into pRH33 NotI

pRH30 pCATCH-m/hβ-cat WT-R (aa 129-781)
pGAD424.1-ß-cat WT-R BamHI x SalI ligated into pCATCH BamHI x XhoI

pRH31 pCATCH-m/hβ-cat D162A (aa 129-781)
pGAD424.1-ß-cat D162A BamHI x SalI ligated into pCATCH BamHI x XhoI

pRH32 pCATCH-m/hβ-cat D164A (aa 129-781)
pGAD424.1-ß-cat D164A BamHI x SalI ligated into pCATCH BamHI x XhoI

pRH33 pBridgeMII-hTcf4 (aa 1-130)
pRH15 HindIII ligated into pBridge HindIII

pRH34 pGAD424.1-mPygo2 (PHD)
pGEX-mPygo2 (PHD) BamHI x XhoI B. ligated into pRH14 BamHI x PstI B.

pRH35 pKB342-Arm^{S10}
pBS-Arm^{S10} NotI B. x Asp718 ligated into pKB342 EcoRV x Asp718

pRH36 pKB342-Arm^{S10} E171A
mutation made in pRH35

pRH37 pKB342-Arm^{S10} D172A
mutation made in pRH35

pRH38 pKB342-Arm^{S10} A303W/ I304W
mutation made in pRH35

pRH39 pKB342-Arm^{S10} K443E
mutation made in pRH35

pRH40 pGAD424-m/hβ-cat 3-C "WT" (aa 129-781) **not in frame**
Cut out BamHI/SalI

pRH41 pGAD424-m/hβ-cat 3-C WT-R (aa 129-781) **not in frame**
Cut out BamHI/SalI

pRH42 pGAD424-m/hβ-cat H260A (aa 129-781) **not in frame**
Cut out BamHI/SalI

pRH43 pGAD424-m/hβ-cat K270A (aa 129-781) **not in frame**
Cut out BamHI/SalI

pRH44 pGAD424-m/hβ-cat R274A (aa 129-781) **not in frame**
Cut out BamHI/SalI

pRH45 pGAD424-m/hβ-cat K292A (aa 129-781) **not in frame**
Cut out BamHI/SalI

pRH46 pGAD424-m/hβ-cat R342A (aa 129-781) **not in frame**
Cut out BamHI/SalI

pRH47 pGAD424-m/hβ-cat K345A (aa 129-781) **not in frame**
Cut out BamHI/SalI

pRH48 pGAD424-m/hβ-cat K354A (aa 129-781) **not in frame**
Cut out BamHI/SalI

pRH49 pGAD424-m/hβ-cat R376A (aa 129-781) **not in frame**
Cut out BamHI/SalI

pRH50 pGAD424-m/hβ-cat W383A (aa 129-781) **not in frame**
Cut out BamHI/SalI

pRH51 pGAD424-m/hβ-cat R386A (aa 129-781) **not in frame**
Cut out BamHI/SalI

pRH52 pGAD424-m/hβ-cat R386A (aa 129-781) **not in frame**
Cut out BamHI/SalI

pRH53 pGAD424-m/hβ-cat K394A (aa 129-781) **not in frame**
Cut out BamHI/SalI

pRH54 pGAD424.1-m/hβ-cat WT (aa 129-781)
Cut out BamHI/SalI

pRH55 pGAD424.1-m/hβ-cat WT-R (aa 129-781)
Cut out BamHI/SalI

pRH56 pGAD424.1-m/hβ-cat R151A (aa 129-781)
Cut out BamHI/SalI

pRH57 pGAD424.1-m/hβ-cat K158A (aa 129-781)
Cut out BamHI/SalI

pRH58 pGAD424.1-m/hβ-cat D162A (aa 129-781)
Cut out BamHI/SalI

pRH59 pGAD424.1-m/hβ-cat E163A (aa 129-781)
Cut out BamHI/SalI

pRH60 pGAD424.1-m/hβ-cat D164A (aa 129-781)
Cut out BamHI/SalI

pRH61 pGAD424.1-m/hβ-cat K170A (aa 129-781)
Cut out BamHI/SalI

pRH62 pGAD424.1-m/hβ-cat K180A (aa 129-781)
Cut out BamHI/SalI

pRH63 pGAD424.1-m/hβ-cat K181A (aa 129-781)
Cut out BamHI/SalI

pRH64 pGAD424.1-m/hβ-cat R190A (aa 129-781)
Cut out BamHI/SalI

pRH65 pGAD424.1-m/hβ-cat R200A (aa 129-781)
Cut out BamHI/SalI

pRH66 pGAD424.1-m/hβ-cat R212A (aa 129-781)
Cut out BamHI/SalI

pRH67 pGAD424.1-m/hβ-cat H223A (aa 129-781)
Cut out BamHI/SalI

pRH68 pGAD424.1-m/hβ-cat R225A (aa 129-781)
Cut out BamHI/SalI

pRH69 pGAD424.1-m/hβ-cat F253A (aa 129-781)
Cut out BamHI/SalI

pRH70 pGAD424.1-m/hβ-cat T257A (aa 129-781)
Cut out BamHI/SalI

pRH71 pGAD424.1-m/hβ-cat H260A (aa 129-781)
Cut out BamHI/SalI

pRH72 pGAD424.1-m/hβ-cat K270A (aa 129-781)
Cut out BamHI/SalI

pRH73 pGAD424.1-m/hβ-cat R274A (aa 129-781)
Cut out BamHI/SalI

pRH74 pGAD424.1-m/hβ-cat K292A (aa 129-781)
Cut out BamHI/SalI

pRH75 pGAD424.1-m/hβ-cat F293A (aa 129-781)
Cut out BamHI/SalI

pRH76 pGAD424.1-m/hβ-cat I296A (aa 129-781)
Cut out BamHI/SalI

pRH77 pGAD424.1-m/hβ-cat W338A (aa 129-781)
Cut out BamHI/SalI

pRH78 pGAD424.1-m/hβ-cat R342A (aa 129-781)
Cut out BamHI/SalI

pRH79 pGAD424.1-m/hβ-cat K345 (aa 129-781)
Cut out BamHI/SalI

pRH80 pGAD424.1-m/hβ-cat K354A (aa 129-781)
Cut out BamHI/SalI

pRH81 pGAD424.1-m/hβ-cat R376A (aa 129-781)
Cut out BamHI/SalI

pRH82 pGAD424.1-m/hβ-cat W383A (aa 129-781)
Cut out BamHI/SalI

pRH83 pGAD424.1-m/hβ-cat R386A (aa 129-781)
Cut out BamHI/SalI

pRH84 pGAD424.1-m/hβ-cat K394A (aa 129-781)
Cut out BamHI/SalI

pRH85 pGAD424.1-m/hβ-cat N426A (aa 129-781)
Cut out BamHI/SalI

pRH86 pGAD424.1-m/hβ-cat L427E (aa 129-781)
Cut out BamHI/SalI

pRH87 pGAD424.1-m/hβ-cat N430A (aa 129-781)
Cut out BamHI/SalI

pRH88 pGAD424.1-m/hβ-cat K435A (aa 129-781)
Cut out BamHI/SalI

pRH89 pGAD424.1-m/hβ-cat R457A (aa 129-781)
Cut out BamHI/SalI

pRH90 pGAD424.1-m/hβ-cat E462A (aa 129-781)
Cut out BamHI/SalI

pRH91 pGAD424.1-m/hβ-cat R469A (aa 129-781)
Cut out BamHI/SalI

pRH92 pGAD424.1-m/hβ-cat H470A (aa 129-781)
Cut out BamHI/SalI

pRH93 pGAD424.1-m/hβ-cat W504A (aa 129-781)
Cut out BamHI/SalI

pRH94 pGAD424.1-m/hβ-cat N516A (aa 129-781)
Cut out BamHI/SalI

pRH95 pGAD424.1-m/hβ-cat WT-R (aa 1-781)
p30 BamHI x PmlI ligated into pRH55 BamHI x PmlI, can be cut out BamHI/SalI

pRH96 pGAD424.1-m/hβ-cat K158A (aa 1-781)
p30 BamHI x PmlI ligated into pRH57 BamHI x PmlI, can be cut out BamHI/SalI

pRH97 pGAD424.1-m/hβ-cat D162A (aa 1-781)
p30 BamHI x PmlI ligated into pRH58 BamHI x PmlI, can be cut out BamHI/SalI

pRH98 pGAD424.1-m/hβ-cat E163A (aa 1-781)
p30 BamHI x PmlI ligated into pRH59 BamHI x PmlI, can be cut out BamHI/SalI

pRH99 pGAD424.1-m/hβ-cat D164A (aa 1-781)
p30 BamHI x PmlI ligated into pRH60 BamHI x PmlI, can be cut out BamHI/SalI

PRH100 pBTM116.2-dE-APC
dE-APC EcoRI x SalI ligated into pBTM116.2 EcoRI x XhoI

pRH101 pBTM116-Pan (aa 1-129)
pTK-Pan EcoRI ligated into pBTM116 EcoRI

pRH102 pGAD424-Arm(myc)-wt (aa 38-806)
p39 EcoRI x NcoI blunt ligated into pGAD424 BamHI blunt

pRH103 pGAD424-Arm(myc)-E171A (aa 38-806)
p40 EcoRI x NcoI blunt ligated into pGAD424 BamHI blunt

pRH104 pGAD424-Arm(myc)-D172A (aa 38-806)
p41 EcoRI x NcoI blunt ligated into pGAD424 BamHI blunt

pRH105 pGAD424-Arm(myc)-A303W/I304W (aa 38-806)
p42 EcoRI x NcoI blunt ligated into pGAD424 BamHI blunt

pRH106 pGAD424-Arm(myc)-K443E (aa 38-806)
p43 EcoRI x NcoI blunt ligated into pGAD424 BamHI blunt

pRH107 pGAD424-hPlakoglobin
p66 EcoRI blunt ligated into pGAD424 SmaI

pRH108 pCATCH-m/hβ-cat-S33Y-wt (aa 1-781)
pGAD424-S33Y-wt BamHI x SalI ligated into pCATCH BamHI x XhoI

pRH109 pCATCH-m/hβ-cat-S33Y-E163A (aa 1-781)
pGAD424-S33Y-E171A BamHI x SalI ligated into pCATCH BamHI x XhoI

pRH110 pCATCH-m/hβ-cat-S33Y-D164A (aa 1-781)
pGAD424-S33Y-D172A BamHI x SalI ligated into pCATCH BamHI x XhoI

pRH111 pcDNA3-CD2-m/hβ-cat-S33Y-wt
pGAD424-S33Y-wt BamHI x SalI ligated into pOP238 BamHI x XhoI

pRH112 pcDNA3-CD2-m/hβ-cat-S33Y-E163A
pGAD424-S33Y-E163A BamHI x SalI ligated into pOP238 BamHI x XhoI

pRH113 pcDNA3-CD2-m/hβ-cat-S33Y-D164A
pGAD424-S33Y-D164A BamHI x SalI ligated into pOP238 BamHI x XhoI

pRH114 pcDNA3.1-KOZAK-m/hβ-cat-S33Y-wt
PCR on pRH108 with mb-cat Asp718 and hb-cat XbaI ligated into pcDNA3.1 Asp718 x XbaI

pRH115 pcDNA3.1-KOZAK-m/hβ-cat-S33Y-E163A
PCR on pRH109 with mb-cat Asp718 and hb-cat XbaI ligated into pcDNA3.1 Asp718 x XbaI

pRH116 pcDNA3.1-KOZAK-m/hβ-cat-S33Y-D164A
PCR on pRH110 with mb-cat Asp718 and hb-cat XbaI ligated into pcDNA3.1 Asp718 x XbaI

pRH117 pcDNA3.1-KOZAK-m/hβ-cat
PCR on pRH95 with mb-cat Asp718 and hb-cat XbaI ligated into pcDNA3.1 Asp718 x XbaI

pRH118 pUAST-CD2-m/hβ-cat-S33Y-wt
pRH111 ApaI blunt x Asp718 ligated into pUAST XbaI blunt x Asp718

pRH119 pUAST-m/hβ-cat-S33Y-wt
pRH114 Asp718 x XbaI ligated into pUAST Asp718 x XbaI

pRH120 pPGS-CMV-CITE-neo-hβ-cat-S33Y-D164A
PCR on p59 with hbcat D164Af and D164Ar XhoI x EcoRI ligated into p59 XhoI x EcoRI

pRH121 pcDNA3.1B-V5-HIS-Lef1
pCR on p54 with m.m.Lef XbaI and m.m.Lef EcoRI ligated into pcDNA3.1B-V5-HIS XbaI x EcoRI

pRH122 pGAD424-Arm-F261D/F301D (aa 38-806)
pKB384-Arm-F261D/F301D DraIII x BssHII ligated into pRH102 DraIII x BssHII

pRH123 pBTM116.2-dEcad (aa 1346-1508) **auto activates**
PCR on T289 fly DNA with dE-cad (EcoRI) and SV40 rev, TOPO cloned, EcoRI x NotI ligated into pBTM116.2 EcoRI x NotI

pRH124 pPGS-CMV-CITE-neo-hβ-cat-S33Y-E163A
PCR on p59 with hbcat E163Af and E163Ar XhoI x EcoRI ligated into p59 XhoI x EcoRI

pRH125 pBTM116-mα-cat (aa 1-750)
p16 EcoRV x BamHI ligated into pBTM116 SmaI x BamHI

pRH126 pBS-dα-cat (aa 1-394)
PCR on EST dα-cat with dα-cat EcoRI and dα-cat SalI ligated into pBS EcoRI x SalI

pRH127 pPGS-CITE-m/hβ-cat-S33Y
pRH114 Asp718 x XbaI blunt ligated into pPGS-CITE+ EcoRI blunt

pRH128 pPGS-CMV-m/hβ-cat-S33Y
pRH114 Asp718 x XbaI blunt ligated into pPGS-CMV+ EcoRI blunt

pRH129 pPGS-CMV-FLAG+
FLAG BamHI & NotI oligo ligated into pPGS-CMV+ BamHI x NotI

pRH130 pUAST-m/hβ-cat-S33Y-D164A
pRH16 Asp718 x XbaI ligated into pUAST Asp718 x XbaI

pRH131 pPGS-CITE-CD2-m/hβ-cat-S33Y
pRH111 Asp718 x ApaI blunt ligated into pPGS-CITE+ EcoRI blunt

pRH132 pcDNA3.1-KOZAK-m/hβ-cat-S33Y-D162A/D164A
Quicksite mutagenesis PCR on pRH116 to introduce D162A using D162A f mbcatD164A and D162A r mbcatD164A, can be cut out with Asp718 x XbaI

pRH133 pAc5.1/V5-HIS-B-KOZAK-m/hβ-cat-
pRH117 Asp718 x XbaI ligated into pRE147 Asp718 x XbaI

pRH134 pcDNA3.1-Gal4-DNA-BD
pRE145 Asp718 x BglII ligated into pcDNA3.1 Asp718 x BamHI

pRH135 pAc5.1/V5-HIS-B-KOZAK-m/hβ-cat-D164A
Quicksite mutagenesis PCR on pRH133 with mbcat D164Af/r to introduce D164A

PRH136 pGAD424.1-m/hβ-cat-R386A (aa 1-781)
Quicksite mutagenesis PCR on pRH95 with hbcat R386Af/r, can be cut out with BamHI x SalI

pRH137 pGAD424.1-m/hβ-cat-Y142A (aa 1-781)
Quicksite mutagenesis PCR on pRH95 with mbcat Y142Af/r, can be cut out with BamHI x SalI

pRH138 pMT-KOZAK-m/hβ-cat
pRH133 Asp718 x XbaI ligated into pMT/V5-HIS-B Asp718 x XbaI

pRH139 pMT-KOZAK-m/hβ-cat-D164A
pRH135 Asp718 x XbaI ligated into pMT/V5-HIS-B Asp718 x XbaI

pRH140 pAc-Gal4BD-ArmR1-4-D172A (aa 156-356)PCR f ArmR1-4S and r ArmR1-4 on p47, cut BglII x BssHII + pRE170 Asp718 x BglII ligated (3-way) into pRE149 Asp718 x BglII

pRH141 pGAD424.1-m/hβ-cat-Y142D (aa 1-781)
Quicksite mutagenesis on pRH95 with mbcat Y142Df/r, can be cut out with BamHI x SalI

pRH142 pAc-Gal4BD-ΔN-Arm (aa 156-843)
PCR on p45 with f ArmR1-4S and Arm MluI cut with BglII x MluI ligated into pAc-Gal4BD BglII x BssHII

pRH143 pGAD424(MCS pRE192)
oligo MCS pRE192 ligated into pGAD424 EcoRI x PstI

pRH144 pCR2.1-mWnt3a
PCR on p75 with mWnt3a f and mWnt3a r, TOPO cloned

pRH145 pGAD424.1-m/hβ-cat-Y142E
Quicksite mutagenesis on pRH95 to introduce Y142E, can be cut out with BamHI x SalI

pRH146 pcDNA3.1-mWnt3a
pRH144 HindIII x EcoRI ligated into pcDNA3.1 HindIII x EcoRI

pRH147 pcDNA3.1-mWnt3a-V5
pRH144 HindIII x XbaI ligated into pcDNA3.1/V5 HindIII x XbaI

pRH148 pCR2.1-hLgs2
PCR on p79 with hLgs2 HD1+2f and hLgs2 HD1+2r, TOPO cloned

pRH149 pBTM116-hLgs2 (HD1+2)
pRH148 EcoRI x NotI ligated into pRE192 EcoRI x NotI

pRH150 pBS-hP300-CH3 (aa 1641-1956) (pointmutation T1924A)
PCR on p78 with hP300 CH3f and hP300 CH3r, cut with BamHI x NotI ligated into pBS BamHI x NotI

pRH151 pCR2.1-mβ-cat (aa 130-781)
PCR on p30 with Bam mß-cat and BGH rev, TOPO cloned

pRH152 pCR2.1-mβ-cat-Y654A (aa 130-781)
Quicksite mutagenesis on pRH151 with mbcat Y654Af/r, can be cut out with BamHI

pRH153 pCR2.1-mβ-cat-F660A (aa 130-781)
Quicksite mutagenesis on pRH151 with mbcat F660Af/r, can be cut out with BamHI

pRH154 pCR2.1-mβ-cat-R661A (aa 130-781)
Quicksite mutagenesis on pRH151 with mbcat R661Af/r, can be cut out with BamHI

pRH155 pCR2.1-mβ-cat-E664A (aa 130-781)
Quicksite mutagenesis on pRH151 with mbcat E664Af/r, can be cut out with BamHI

pRH156 pcDNA3.1-NLS/V5-His
Oligo NLS ligated into pcDNA3.1/V5-His Asp718 x BamHI

pRH157 pGAD424-mβ-cat-wt (aa 130-781)
pRH151 BamHI ligated into pRH143 BamHI

pRH158 pGAD424-mβ-cat-Y654A (aa 130-781)
pRH152 BamHI ligated into pRH143 BamHI

pRH159 pGAD424-mβ-cat-F660A (aa 130-781)
pRH153 BamHI ligated into pRH143 BamHI

pRH160 pGAD424-mβ-cat-R661A (aa 130-781)
pRH154 BamHI ligated into pRH143 BamHI

pRH161 pGAD424-mβ-cat-E664A (aa 130-781)
pRH155 BamHI ligated into pRH143 BamHI

pRH162 pBTM116-hP300-CH3 (aa 1641-1959)
pRH150 BamHI x NotI ligated into pRE192 BamHI x NotI

pRH163 pcDNA3.1/V5-mWnt3a (no V5 tag)
pRH144 HindIII x EcoRI ligated into pcDNA3.1/V5-His HindIII x EcoRI

pRH164 pBTM116-mCBP (1-188) **auto activates**
p76 BamHI x ApaI blunt ligated into pRE192 BamHI x PstI blunt

pRH165 pBTM116-mCBP (1-359) **auto activates**
p76 BamHI x EcoRV ligated into pRE192 BamHI x PstI blunt

pRH166 pcDNA3.1-Gal4-mβ-cat-wt (aa 130-781) **stop between Gal4&mß-cat**
pRH157 BamHI blunt ligated into pRH134 EcoRI blunt

pRH167 pcDNA3.1-Gal4-mβ-cat-Y654A (aa 130-781) **stop between Gal4&mß-cat**
pRH158 BamHI blunt ligated into pRH134 EcoRI blunt

pRH168 pcDNA3.1-Gal4-mβ-cat-F660A (aa 130-781) **stop between Gal4&mß-cat**
pRH159 BamHI blunt ligated into pRH134 EcoRI blunt

pRH169 pcDNA3.1-Gal4-mβ-cat-R661A (aa 130-781) **stop between Gal4&mß-cat**
pRH160 BamHI blunt ligated into pRH134 EcoRI blunt

pRH170 pcDNA3.1-Gal4-mβ-cat-E664A (aa 130-781) **stop between Gal4&mß-cat**
pRH161 BamHI blunt ligated into pRH134 EcoRI blunt

pRH171 pcDNA3.1-Gal4BD.2
PCR on pRH134 with Gal4BD Asp718 f and Gal4BD BamHI r ligated into pcDNA3.1 Asp718 x BamHI

pRH172 pBS-mβ-cat-ΔC (aa 1-673)
PCR on p30 with mbcatΔC NotI and Bam f mbcat ligated into pBS BamHI x Not

pRH173 pcDNA3.1-myc
Oligo Asp718 myc BamHI ligated into pcDNA3.1 Asp718 x BamHI

pRH174 pBTM116-mβ-cat (aa 536-781) **auto activates**
pRH157 SpeI blunt x SalI ligated into pBTM116 BamHI blunt x SalI

pRH175 pcDNA3.1-Gal4BD.2-ΔN-mβ-cat-wt (aa 130-781)
pRH157 BamHI x SmaI ligated into pRH171 BamHI x EcoRV

pRH176 pcDNA3.1-Gal4BD.2-ΔN-mβ-cat-Y654A (aa 130-781)
pRH158 BamHI x SmaI ligated into pRH171 BamHI x EcoRV

pRH177 pcDNA3.1-Gal4BD.2-ΔN-mβ-cat-F660A (aa 130-781)
pRH159 BamHI x SmaI ligated into pRH171 BamHI x EcoRV

pRH178 pcDNA3.1-Gal4BD.2-ΔN-mβ-cat-R661A (aa 130-781)
pRH160 BamHI x SmaI ligated into pRH171 BamHI x EcoRV

pRH179 pcDNA3.1-Gal4BD.2-ΔN-mβ-cat-E664A (aa 130-781)
pRH161 BamHI x SmaI ligated into pRH171 BamHI x EcoRV

pRH180 pcDNA3.1-myc-ΔN-mβ-cat-wt (aa 130-781)
pRH157 BamHI x SmaI ligated into pRH173 BamHI x EcoRV

pRH181 pcDNA3.1-myc-ΔN-mβ-cat-Y654A (aa 130-781)
pRH158 BamHI x SmaI ligated into pRH173 BamHI x EcoRV

pRH182 pcDNA3.1-myc-ΔN-mβ-cat-F660A (aa 130-781)
pRH159 BamHI x SmaI ligated into pRH173 BamHI x EcoRV

pRH183 pcDNA3.1-myc-ΔN-mβ-cat-R661A (aa 130-781)
pRH160 BamHI x SmaI ligated into pRH173 BamHI x EcoRV

pRH184 pcDNA3.1-myc-ΔN-mβ-cat-E664A (aa 130-781)
pRH161 BamHI x SmaI ligated into pRH173 BamHI x EcoRV

pRH185 pcDNA3.1-GAL4BD.2-ΔN-mβ-cat-D164A (aa 130-781)
Quicksite mutagenesis on pRH175 with mbcat D164Af/r, can be cut out with BamHI x XbaI

pRH186 pBS-Arm(myc) (contains tub-trailer)
p45 Asp718 x XbaI ligated into pBS Asp718 x XbaI

pRH187 pBS-Arm^{S10} (myc) (contains tub-trailer)
pDA720 Asp718 x XbaI ligated into pBS Asp718 x XbaI

pRH188 pcDNA3-NLS-HA-dLgsΔHD1
pTK98 AgeI x XhoI ligated into pOP244 AgeI x XhoI

pRH189 pcDNA3.1-NLS-mβ-cat-ΔC-V5/His (aa 1-673)
pRH172 BamHI x NotI ligated into pRH156 BamHI x NotI

pRH190 pMZ55-mCBP (aa 1-270)
pRH165 Asp718 blunt x BamHI ligated into pMZ55 HindIII blunt x BamHI

pRH191 ptub-Arm(myc)-Y150A
Quicksite mutagenesis on pRH186 with Arm Y150Af1/r1, cut Asp718 x XbaI ligated into pOP118 Asp718 x XbaI

pRH192 pUAST-Arm^{S10} (myc)-Y150A
Quicksite mutagenesis on pRH187 with Arm Y150Af1/r1, cut Asp718 x XbaI ligated into pUAST Asp718 x XbaI

pRH193 pcDNA3.1-GAL4BD.2-ΔN-mβ-cat-F660A/E664A (aa 130-781)
Quicksite mutagenesis on pRH179 with mbcat F660A on E664Af/r, cut BamHI x XbaI ligated into pRH171 BamHI x XbaI

pRH194 pcDNA3.1-GAL4BD.2-mβ-cat-S33Y-wt (aa 1-781)
pRH111 BamHI x BbrPI ligated into pRH175 BamHI x BbrPI

pRH195 pcDNA3.1-GAL4BD.2-mβ-cat-S33Y-Y654A (aa 1-781)
pRH111 BamHI x BbrPI ligated into pRH176 BamHI x BbrPI

pRH196 pcDNA3.1-GAL4BD.2-mβ-cat-S33Y-F660A (aa 1-781)
pRH111 BamHI x BbrPI ligated into pRH177 BamHI x BbrPI

pRH197 pcDNA3.1-GAL4BD.2-mβ-cat-S33Y-R661A (aa 1-781)
pRH111 BamHI x BbrPI ligated into pRH178 BamHI x BbrPI

pRH198 pcDNA3.1-GAL4BD.2-mβ-cat-S33Y-E664A (aa 1-781)
pRH111 BamHI x BbrPI ligated into pRH179 BamHI x BbrPI

pRH199 pcDNA3.1-GAL4BD.2-mβ-cat-S33Y-D164A (aa 1-781)
pRH111 BamHI x BbrPI ligated into pRH185 BamHI x BbrPI

pRH200 ptub-NLS-HA-dLgs
pOP244 Asp718 x XbaI ligated into pOP118 Asp718 x XbaI

pRH201 ptub-NLS-HA-dLgsΔHD1
pRH188 Asp718 x XbaI ligated into pOP118 Asp718 x XbaI

pRH202 pBS-NLS-HA-tub trailer
Oligo Asp718 NLS BssHII ligated into pMZ55 Asp718 NLS BssHII

pRH203 pcDNA3.1-HA-mCBP (aa 1-270)
pRH190 Asp718 x XbaI ligated into pcDNA3.1 Asp718 x XbaI

pRH204 pBS-NLS-HA-BCL9NΔHD1 (aa 1-732)
PCR with BglII K. BCL9 and BCL9 732 S. NheI on pTK118 cut with BglII x NotI ligated into pRH202 BamHI x NotI

pRH205 pcDNA3.1-GAL4BD.2-mβ-cat-S33Y-Y142A
pRH137 BbrPI x BsmBI ligated into pRH194 BbrPI x BsmBI

pRH206 pBS-NLS-HA-BCL9N (aa 1-732)
PCR with BglII K. BCL9 and BCL9 732 S. NheI on p55 cut with BglII x NotI ligated into pRH202 BamHI x NotI

pRH207 pcDNA3.1-GAL4DBD.2-mβ-cat-R8-C-wt (aa 410-781)
Oligo BamHI BstEII ligated into pRH175 BamHI x BstEII

pRH208 pcDNA3.1-GAL4BD.2-mβ-cat-S33Y-Y142E
pRH145 BbrPI x BsmBI ligated into pRH194 BbrPI x BsmBI

pRH209 pPacPL-NLS-HA-BCL9NΔHD1 (aa 1-732)
pRH204 cut with Asp718 x NotI ligated into pRE398 Asp718 x NotI

pRH210 pcDNA3.1-NLS-HA-BCL9NΔHD1 (aa 1-732)
pRH204 cut with Asp718 x NotI ligated into pcDNA3.1 Asp718 x NotI

pRH211 pcDNA3.1-NLS-HA-BCL9N (aa 1-732)
pRH206 cut with Asp718 x NotI ligated into pcDNA3.1 Asp718 x NotI

pRH212 pPacPL-NLS-HA-BCL9N (aa 1-732)
pRH206 cut with Asp718 x NotI ligated into pRE398 Asp718 x NotI

pRH213 pcDNA3.1-GAL4BD.2-mβ-cat-S33Y-Y142D
pRH141 BbrPI x BsmBI ligated into pRH194 BbrPI x BsmBI

pRH214 pDU10-Ire1ΔTM (aa 555-1115)
pDU62 cut with NotI x SalI ligated into pDU10 NotI x SalI

pRH215 pDU12-Ire1ΔTM (aa 555-1115)
pDU62 cut with NotI x SalI ligated into pDU12 NotI x SalI

pRH216 pBS-mCBP (aa 1-270)
PCR on pRH203 with mCBP 316 NotI r and mCBP XbaI f, cut with XbaI x NotI ligated into pBS XbaI x NotI

pRH217 pBS-BCL9 (aa 199-392)
PCR on p55 with BCL9 199 Xba f and BCL9 392 Not r, cut with XbaI x NotI ligated into pBS XbaI x NotI

pRH218 pGEX-KG-ΔN-mß-cat (aa 130-781)
pRH175 BamHI x XbaI ligated into pGEX-KG BamHI x XbaI

pRH219 pBS-hTCF4 (aa 1-130)
PCR on pRH14 with hTCF4 Xba f and hTCF4 Not r, cut with XbaI x NotI ligated into pBS XbaI x NotI

pRH220 ptub-NLS-HA-LgsN (1-768)
pMZ55 BamHI x XbaI ligated into pRH200 BglII x XbaI

pRH221 pcDNA3.1-GAL4DBD.2-mβ-cat-R8-C-Y654A (aa 410-781)
pRH176 BstEII x XbaI ligated into pRH207 BstEII x XbaI

pRH222 pcDNA3.1-GAL4DBD.2-mβ-cat-R8-C-F660A (aa 410-781)
pRH177 BstEII x XbaI ligated into pRH207 BstEII x XbaI

pRH223 pcDNA3.1-GAL4DBD.2-mβ-cat-R8-C-R661A (aa 410-781)
pRH178 BstEII x XbaI ligated into pRH207 BstEII x XbaI

pRH224 pcDNA3.1-GAL4DBD.2-mβ-cat-R8-C-E664A (aa 410-781)
pRH179 BstEII x XbaI ligated into pRH207 BstEII x XbaI

pRH225 pDU10-Ire1ΔTM-mCBP (aa 1-270)
pRH216 XbaI x NotI ligated into pRH214 XbaI x NotI

pRH226 pDU10-Ire1ΔTM-BCL9 (aa 199-392)
pRH217 XbaI x NotI ligated into pRH214 XbaI x NotI

pRH227 pPacPL-HA-BCL9N (aa 1-732)
Oligo Asp718 ATG BssHII ligated into pRH212 Asp718 x BssHII

pRH228 pPacPL-HA-BCL9NΔHD1 (aa 1-732)
Oligo Asp718 ATG BssHII ligated into pRH209 Asp718 x BssHII

pRH229 pDU10-Ire1ΔTM-hTCF4 (aa 1-130)
pRH219 XbaI x NotI ligated into pRH214 XbaI x NotI

pRH230 ptub-NLS-HA-LgsN-ΔHD1 (aa 1-768)
pRH201 BamHI ligated into pRH220 BamHI

pRH231 pcDNA3.1-HA (wg leader-HA)
pMZ55 Asp718 x BamHI ligated into pcDNA3.1 Asp718 x BamHI

pRH232 pPacPL-Arm-HA-NLS
pRH186 Asp718 x ApaI ligated into pRE367 Asp718 x ApaI

pRH233 pcDNA3.1-GAL4BD.2-mβ-cat-S33Y-D164A/Y654A (aa 1-781)
pRH199 SpeI ligated into pRH221 SpeI

pRH234 pcDNA3.1-HA-hICAT
pGEX-4T-3-hICAT BamHI x EcoRI ligated into pRH231 BamHI x EcoRI

pRH235 pcDNA3.1-HA-hP300-CH3 (1710-1891)
pGEX-4T-1-hP300-CH3 BamHI x NotI ligated into pRH231 BamHI x NotI

pRH236 ptub-LgsN (aa 1-768)
pMZ55 BamHI x XbaI ligated into pOP212 BglII x XbaI

pRH237 pcDNA3.1-GAL4DBD.2-mβ-cat-R8-C-Y654E (aa 410-781)
Quicksite mutagenesis on pRH207 with mbcat Y654Ef/r, can be cut out with BamHI x XbaI

pRH238 pcDNA3.1-GAL4DBD.2-mβ-cat-R8-C-F660A/R661A (aa 410-781)
Quicksite mutagenesis on pRH222 with mbcat R661A on F660Af/r, can be cut out with BamHI x XbaI

pRH239 pcDNA3.1-NLS-HA-LgsN (aa 1-768)
pRH220 Asp718 x XbaI ligated into pcDNA3.1 Asp718 x XbaI

pRH240 pcDNA3.1-NLS-HA-LgsNΔHD1 (aa 1-768)
pRH230 Asp718 x XbaI ligated into pcDNA3.1 Asp718 x XbaI

pRH241 pBTM116-hICAT
pGEX-4T-3-hICAT BamHI x NotI ligated into pRE192 BamHI x NotI

pRH242 pBTM116-hP300-CH3 (aa 1710-1891)
pGEX-4T-1-hP300-CH3 BamHI x NotI ligated into pRE192 BamHI x NotI

pRH243 pcDNA3.1-NLS-Arm
pRH232 Asp718 x XbaI ligated into pcDNA3.1 Asp718 x XbaI

pRH244 pcDNA3.1-HA-LgsN (aa 1-768)
pOP221 Asp718 x PinAI ligated into pRH239 Asp718 x PinAI

pRH245 pcDNA3.1-HA-LgsNΔHD1 (aa 1-768)
pOP221 Asp718 x PinAI ligated into pRH240 Asp718 x PinAI

pRH246 pGAD424-m/hβ-cat-N-R5-wt (aa 1–356)
pRH95 NgoMIV blunt x Sal I blunt, religated

pRH247 pGAD424-m/hβ-cat-N-R5-Y142A (aa 1–356)
pRH137 NgoMIV blunt x Sal I blunt, religated

pRH248 pGAD424-m/hβ-cat-N-R5-D164A (aa 1–356)
pRH99 NgoMIV blunt x Sal I blunt, religated

pRH249 pcDNA3-CD2-mβ-cat-S33Y-Y142A
pRH205 BamHI x NotI ligated into pOP238 BamHI x NotI

pRH250 pBS-hPygo2-Δ74-78
pRE201 BglII x BssHII ligated into pRE344 BglII x BssHII

pRH251 pBS-hPygo2-ΔThomas
pRE202 BglII x BssHII ligated into pRE344 BglII x BssHII

pRH252 ptub-HA-LgsN (aa 1-768)
pRH244 Asp718 x XbaI ligated into pOP118 Asp718 x XbaI

pRH253 ptub-HA-LgsNΔHD1 (aa 1-768)
pRH245 Asp718 x XbaI ligated into pOP118 Asp718 x XbaI

pRH254 pcDNA3.1-HA-mβ-cat-wt (aa 410-781)
pRH207 BamHI x NotI ligated into pRH231 BamHI x NotI

pRH255 pcDNA3.1-HA-mβ-cat-Y654A (aa 410-781)
pRH221 BamHI x NotI ligated into pRH231 BamHI x NotI

pRH256 pcDNA3.1-HA-mβ-cat-F660A (aa 410-781)
pRH222 BamHI x NotI ligated into pRH231 BamHI x NotI

pRH257 pcDNA3.1-HA-mβ-cat-R661A (aa 410-781)
pRH223 BamHI x NotI ligated into pRH231 BamHI x NotI

pRH258 pcDNA3.1-HA-mβ-cat-E664A (aa 410-781)
pRH224 BamHI x NotI ligated into pRH231 BamHI x NotI

pRH259 pcDNA3.1-HA-LgsN2-eGFP (aa 1-782)
pRE356 XhoI x XbaI ligated into pOP221 XhoI x XbaI

pRH260 pcDNA3.1-NLS-HA-LgsN2-eGFP (aa 1-782)
pRE356 XhoI x XbaI ligated into pOP244 XhoI x XbaI

pRH261 pcDNA3.1-HA-LgsN2-ΔHD1-eGFP (aa 1-782)
pRH245 ScaI ligated into pRH259 ScaI

pRH262 pcDNA3.1-NLS-HA-LgsN2-ΔHD1-eGFP (aa 1-782)
pRH240 ScaI ligated into pRH259 ScaI

pRH263 pPacPL-HA-LgsN2-eGFP (aa 1-782)
pRH259 Asp718 x XbaI blunt ligated into pRE262 Asp718 x NotI blunt

pRH264 pPacPL-NLS-HA-LgsN2-eGFP (aa 1-782)
pRH260 Asp718 x XbaI blunt ligated into pRE262 Asp718 x NotI blunt

pRH265 pPacPL-HA-LgsN2-ΔHD1-eGFP (aa 1-782)
pRH261 Asp718 x XbaI blunt ligated into pRE262 Asp718 x NotI blunt

pRH266 pPacPL-NLS-HA-LgsN2-ΔHD1-eGFP (aa 1-782)
pRH262 Asp718 x XbaI blunt ligated into pRE262 Asp718 x NotI blunt

pRH267 pUAST-CD2-mβ-cat-S33Y-Y142A
pRH249 Asp718 x NotI blunt ligated into pUAST Asp718 x XbaI blunt

pRH268 pcDNA3.1-hPygo2Δ74-78
pRH250 XbaI blunt x NotI ligated into pcDNA3.1 EcoRV x NotI

pRH269 pcDNA3.1-hPygo2ΔThomas
pRH251 XbaI blunt x NotI ligated into pcDNA3.1 EcoRV x NotI

pRH270 pPacPL-FLAG-mBCL9.2-FL
p85 NheI x XbaI ligated into pRE402 XbaI x NheI

pRH271 pPacPL-FLAG-mBCL9.2-Δ138-173
p86 NheI x XbaI ligated into pRE402 XbaI x NheI

pRH272 pPacPL-FLAG-mBCL9.2-ΔPyBD
p87 NheI x XbaI ligated into pRE402 XbaI x NheI

pRH273 pBTM116-HA-LgsN (aa 1-782)
pRH263 EcoRV x XhoI ligated into pBTM116.2 SmaI x XhoI

pRH274 pGAD424-mß-catΔC (aa 1-673)
pRH172 BamHI x NotI ligated into pRH143 BamHI x NotI

pRH275 pcDNA3.1-G4DBD-mβ-cat-S33Y-ΔC-V5/His
pRH194 SpeI ligates into pRH189 SpeI

pRH276 pcDNA3.1-G4DBD-mβ-cat-S33Y-D164A-ΔC-V5/His
pRH199 SpeI ligates into pRH189 SpeI

pRH277 pBS-mβ-cat-R1-4-wt (aa 141-306)
PCR on p30 with mß-catR1 f and mß-catR4 r ligated into pBS BamHI x NotI

pRH278 pBS-mβ-cat-R1-4-D164A (aa 141-306)
PCR on pRH199 with mß-catR1 f and mß-catR4 r ligated into pBS BamHI x NotI

pRH279 pBS-mβ-cat-R1-4-Y142A (aa 141-306)
PCR on pRH205 with mß-catR1-Y142A f and mß-catR4 r ligated into pBS BamHI x NotI

pRH280 pcDNA3.1-NLS-G4DBD-BCL9-HA (tub trailer)
pTK006 SalI blunt x NotI ligated into pRH312 EcoRV x NotI

pRH281 pcDNA3.1-NLS-G4DBD-BCL9ΔHD1-HA (tub trailer)
pTK136 SalI blunt x NotI ligated into pRH312 EcoRV x NotI

pRH282 pcDNA3.1-NLS-G4DBD-FLAG-mBCL9.2-FL
p85 NheI x EcoRI ligated into pRH312 NheI x EcoRI

pRH283 pcDNA3.1-NLS-G4DBD-FLAG-mBCL9.2-Δ138-173
p86 NheI x EcoRI ligated into pRH312 NheI x EcoRI

pRH284 pcDNA3.1-NLS-G4DBD-FLAG-mBCL9.2-ΔPyBD
p87 NheI x EcoRI ligated into pRH312 NheI x EcoRI

pRH285 pBS-Arm(myc)-D172A (contains tub-trailer)
pDA725 Asp718 x XbaI ligated into pBS Asp718 x XbaI

pRH286 pBS-Arm^{S10}-D172A (myc) (contains tub-trailer)
pDA722 Asp718 x XbaI ligated into pBS Asp718 x XbaI

pRH287 pBS-Arm-ΔC-HA (aa 1-690)
pRE008 ApaI x XbaI ligated into pRH186 ApaI x XbaI

pRH288 pBS-Arm^{S10}-ΔC-HA (aa 1-690)
pRE008 ApaI x XbaI ligated into pRH187 ApaI x XbaI

pRH289 pBS-Arm-D172A-ΔC-HA (aa 1-690)
pRE008 ApaI x XbaI ligated into pRH285 ApaI x XbaI

pRH290 pBS-Arm^{S10}-D172A-ΔC-HA (aa 1-690)
pRE008 ApaI x XbaI ligated into pRH286 ApaI x XbaI

pRH291 pGAD424-mβ-cat-R1-4-wt (aa 141-306)
pRH277 BamHI x NotI ligated into pRH143 BamHI x NotI

pRH292 pGAD424-mβ-cat-R1-4-D164A (aa 141-306)
pRH278 BamHI x NotI ligated into pRH143 BamHI x NotI

pRH293 pGAD424-mβ-cat-R1-4-Y142A (aa 141-306)
pRH279 BamHI x NotI ligated into pRH143 BamHI x NotI

pRH294 ptub-Arm-ΔC-HA (aa 1-690)
pRH287 Asp718 x XbaI ligated into pOP118 Asp718 x XbaI

pRH295 ptub-Arm-D172A-ΔC-HA (aa 1-690)
pRH289 Asp718 x XbaI ligated into pOP118 Asp718 x XbaI

pRH296 pUAST-Arm^{S10}-ΔC-HA (aa 1-690)
pRH288 Asp718 x XbaI ligated into pUAST Asp718 x XbaI

pRH297 pUAST-Arm^{S10}-D172A-ΔC-HA (aa 1-690)
pRH290 Asp718 x XbaI ligated into pUAST Asp718 x XbaI

pRH298 pGAD424.1-m/hβ-cat-Y142F
Quicksite mutagenesis PCR on pRH95 with mbcat Y142Ff/r, can be cut out with BamHI x SalI

pRH299 pPacPL-MCS.2
Oligo MCS.2 ligated into pRE398 BamHI (destroyed) x NotI

pRH300 pBS-Arm^{S10}-ΔC-myc (aa 1-690)
oligo SpeI myc EcoRI ligated into pRH288 SpeI x EcoRI

pRH301 pBS-Arm^{S10}-D172A-ΔC-myc (aa 1-690)
oligo SpeI myc EcoRI ligated into pRH290 SpeI x EcoRI

pRH302 pcDNA3.1-HA-mPygo2-PHD (aa 276-406)
pRH34 BamHI x XhoI ligated into pRH231 BamHI x XhoI

pRH303 pBS-Arm-ΔC-myc
pRH287 Asp718 x StuI ligated into pRH300 Asp718 x StuI

pRH304 pBS-Arm-D172A-ΔC-myc
pRH289 Asp718 x StuI ligated into pRH300 Asp718 x StuI

pRH305 pcDNA3.1-NLS-GAL4BD.2-mβ-cat-S33Y-wt (aa 1-781)
pRE492 Asp718 x BglII ligated into pRH194 Asp718 x BamHI

pRH306 pcDNA3.1-NLS-GAL4BD.2-mβ-cat-S33Y-D164A (aa 1-781)
pRE492 Asp718 x BglII ligated into pRH199 Asp718 x BamHI

pRH307 pcDNA3.1-NLS-GAL4BD
pRE492 Asp718 x HpaI ligated into pRH171 Asp718 x HpaI

pRH308 pcDNA3.1-NLS-GAL4BD-HA (stop after HA)
pRE492 Asp718 x HpaI ligated into pRE428 Asp718 x HpaI

pRH309 pPacPL-BCL9-HA
pTK006 HindIII blunt x SalI ligated into pRH299 EcoRV x XhoI

pRH310 ptub-ArmΔC-myc
pRH303 Asp718 x XbaI ligated into pOP118 Asp718 x XbaI

pRH311 ptub-ArmΔC-D172A-myc
pRH304 Asp718 x XbaI ligated into pOP118 Asp718 x XbaI

pRH312 pcDNA3.1-NLS-G4DBD-MCS new
oligo BamHI MCS XbaI ligated into pRH307 Asp718 x XbaI

pRH313 pPacPL-BCL9ΔHD1-HA
pTK136 Asp718 ligated into pRH309 Asp718

pRH314 pcDNA3.1-BCL9-HA
pTK006 SalI blunt x NotI ligated into pcDNA3.1 EcoRV x NotI

pRH315 pcDNA3.1-BCL9ΔHD1-HA
pTK106 SalI blunt x NotI ligated into pcDNA3.1 EcoRV x NotI

pRH316 pBS-Arm^{S10}-ΔC-NLS-HA
pRE008 ApaI x SpeI ligated into pRE034 ApaI x SpeI

pRH317 pPacPL-Arm-D172A-NLS-HA
pRH289 Asp718 x ApaI ligated into pRE367 Asp718 x ApaI

pRH318 pBS-Arm-ΔC-wt-NLS-HA (contains tub trailer)
pRH186 Asp718 x ApaI ligated into pRH316 Asp718 x ApaI

pRH319 pBS-Arm-ΔC-D172A-NLS-HA (contains tub trailer)
pRH285 Asp718 x ApaI ligated into pRH316 Asp718 x ApaI

pRH320 pPacPL-Arm-wt-ΔC-NLS-HA (aa 1-690)
pRH318 Asp718 x XbaI ligated into pRH299 Asp718 x XbaI

pRH321 pPacPL-Arm-D172A-ΔC-NLS-HA (aa 1-690)
pRH319 Asp718 x XbaI ligated into pRH299 Asp718 x XbaI

pRH322 pBS-mβ-cat-cat w/o STOP (aa 1-781)
PCR on p30 with mß-cat-Asp718 and mß-cat XhoI r ligated into pBS Asp718 x XhoI

pRH323 pUAST-Arm^{S10}-ΔC-myc
pRH300 Asp718 x XbaI ligated into pUAST Asp718 x XbaI

pRH324 pUAST-Arm^{S10}-D172A-ΔC-myc
pRH301 Asp718 x XbaI ligated into pUAST Asp718 x XbaI

pRH325 pBS-hP300
p77 HindIII x NotI ligated into pBS HindIII x NotI

pRH326 pBS-hP300-HA
p78 HindIII x NotI ligated into pBS HindIII x NotI

pRH327 pBS-hPygo2-ΔNHD
pRE329 BglII x NotI ligated into pRE344 BglII x NotI

pRH328 pcDNA3.1-hPygo2-ΔNHD
pRH327 XbaI blunt x NotI ligated into pRE319 Asp718 x NotI

pRH329 pPacPL-V5
Oligo Asp718 V5 NheI ligated into pRH299 Asp718 x NheI

pRH330 pPacPL-FLAG
Oligo Asp718 FLAG NheI ligated into pRH299 Asp718 x NheI

pRH331 pBS-hP300 (aa 1-580)
pRH325 EcoRV x BstBI blunt religated

pRH332 pBS-hP300 (aa 1-1257)
pRH325 EcoRV x BglII blunt religated

pRH333 pBS-hP300 (aa 1-1944)
pRH325 EcoRV x PmlI religated

pRH334 pCR2.1-eGFP (without Kozak and ATG)
PCR on pRE365 with XhoI GFP f and eGFP STOP EcoRI, TOPO cloned

pRH335 pBS-HR1
PCR m. gen. DNA with Spe Pac HR1 f and Not (mut) HR1 r1, ligated into pBS SpeI x NotI

pRH336 pBS-HR2a
PCR m. gen. DNA with Eco Asc HR2a f1 and Xho HR2a r, ligated into pBS EcoRI x XhoI

pRH337 pBS-HR2b
PCR m. gen. DNA with Xho HR2b f and Apa HR2b r, ligated into pBS XhoI x ApaI

pRH338 pBS-HR2c
PCR m. gen. DNA with Apa HR2c f and Asp718 Pme HR2c r, ligated into pBS ApaI x Asp718

PRH339 pcDNA3.1-mβ-cat-eGFP
pRH322 Asp718 x XhoI & pRH334 XhoI x NotI ligated into pcDNA3.1 Asp718 x NotI

pRH340 pTargetVector-HR1
pRH335 PacI x NotI ligated into pT.V. PacI x NotI

pRH341 pBS-HR2
pRH337 XhoI x ApaI & pRH338 ApaI x Asp718 ligated into pRH336 XhoI x Asp718

pRH342 pMZ55-FLAG-BCL9.2-FL-tub trailer (intermediate)
p85 HindIII x EcoRI ligated into pMZ55 HindIII x EcoRI

pRH343 pMZ55- FLAG-BCL9.2-ΔPyBD-tub trailer (intermediate)
p87 HindIII x EcoRI ligated into pMZ55 HindIII x EcoRI

pRH344 pcDNA3.1-NLS-G4DBD-mβ-cat-S33Y-ΔC-V5/His
pRH305 Asp718 x BbrPI ligated into pRH275 Asp718 x BbrPI

pRH345 pcDNA3.1-NLS-G4DBD-mβ-cat-S33Y-D164A-ΔC-V5/His
pRH305 Asp718 x BbrPI ligated into pRH276 Asp718 x BbrPI

pRH346 pPacPL-FLAG-mβ-cat-S33Y-wt
pRH194 BamHI x XbaI ligated into pRH330 BamHI x XbaI

pRH347 pPacPL-FLAG-mβ-cat-S33Y-D164A
pRH199 BamHI x XbaI ligated into pRH330 BamHI x XbaI

pRH348 pPacPL-FLAG-mβ-cat-S33Y-Y142A
pRH205 BamHI x XbaI ligated into pRH330 BamHI x XbaI

pRH349 pPacPL-FLAG-mβ-cat-S33Y-Y142E
pRH208 BamHI x XbaI ligated into pRH330 BamHI x XbaI

pRH350 pPacPL-FLAG-mβ-cat-S33Y-Y142D
pRH213 BamHI x XbaI ligated into pRH330 BamHI x XbaI

pRH351 pcDNA3.1-mβ-cat-S33Y-eGFP
pRH114 Asp718 x BbrPI ligated into pRH339 Asp718 x BbrPI

pRH352 pTargetVector-HR1-HR2
pRH341 PmeI x AscI blunt ligated into pRH340 PmeI

pRH353 pBS-HR3
PCR m. gen. DNA with EcoRI PacI HR3 f.1 and Not HR3 r.1, ligated into pBS EcoRI x NotI

pRH354 pBS-HR4a
PCR m. gen. DNA with Sal Asc HR4a f and EcoRI HR4a r, ligated into pBS SalI x EcoRI

pRH355 pBS-HR4b
PCR m. gen. DNA with EcoRI HR4b f and XmaI HR4b r, ligated into pBS EcoRI x XmaI

pRH356 pBS-HR4c
PCR m. gen. DNA with XmaI HR4c f.1 and XbaI HR4c r.1, ligated into pBS XmaI x XbaI

pRH357 pBS-HR4d
PCR m. gen. DNA with XbaI HR4d f and Not Pme HR4d r, ligated into pBS XbaI x NotI

pRH358 ptub-FLAG-BCL9.2-FL
pRH342 HindIII blunt x XbaI ligated into pOP118 Asp718 x XbaI

pRH359 ptub-FLAG-BCL9.2-ΔPyBD
pRH343 HindIII blunt x XbaI ligated into pOP118 Asp718 x XbaI

pRH360 pTargetVector-HR3
pRH353 PacI x NotI ligated into pT.V. PacI x NotI

pRH361 pBS-HR4a-4b
pRH355 EcoRI x XbaI ligated into pRH354 EcoRI x XbaI

pRH362 pBS-HR4c-4d
pRH357 XbaI x NotI ligated into pRH356 XbaI x NotI

pRH363 pPacPL-V5-dCBP **stop in between V5 and dCBP**
p80 BstBI x NotI ligated into pRH329 BstBI x NotI

pRH364 pPacPL-FLAG-dCBP **stop in between FLAG and dCBP**
p80 BstBI x NotI ligated into pRH330 BstBI x NotI

pRH365 pBS-HR4
pRH361 XmaI x NotI ligated into pRH362 XmaI x NotI

pRH366 pPGS-CMV-CITE-NEO-mβ-cat-S33Y-eGFP
pRH351 Asp718 x XbaI blunt ligated into pPGS-CMV- EcoRI blunt

pRH367 pBS-HR1+
PCR m. gen. DNA with Spe Pac HR1f test and Not (mut) HR1 r1, ligated into pBS SpeI x NotI

pRH368 pBS-HR3+
PCR m. gen. DNA with EcoRI PacI HR3f test and Not HR3 r.1, ligated into pBS EcoRI x NotI

pRH369 pcDNA3.1-HA-hP300-CH1 (aa 301-388)
PCR with Bam hP300 900f and Xba hP300 r, ligated into pRH231 BamHI x XbaI

pRH370 pcDNA3.1-HA-hP300 (aa 1-388)
PCR with Bam hP300 f and Xba hP300 r, ligated into pRH231 BamHI x XbaI

pRH371 pGEX-4T2-hBCL9.2 (HD1 + 2)
pRH148 BamHI x NotI ligated into pGEX-4T2 BamHI x NotI

pRH372 pcDNA3.1-mβ-cat-S33Y-wt
pRH346 Asp718 x XbaI ligated into pcDNA3.1 Asp718 x XbaI

pRH373 pcDNA3.1-mβ-cat-S33Y-D164A
pRH347 Asp718 x XbaI ligated into pcDNA3.1 Asp718 x XbaI

pRH374 pcDNA3.1-mβ-cat-S33Y-Y142A
pRH348 Asp718 x XbaI ligated into pcDNA3.1 Asp718 x XbaI

pRH375 pcDNA3.1-mβ-cat-S33Y-Y142E
pRH349 Asp718 x XbaI ligated into pcDNA3.1 Asp718 x XbaI

pRH376 pcDNA3.1-mβ-cat-S33Y-Y142D
pRH350 Asp718 x XbaI ligated into pcDNA3.1 Asp718 x XbaI

pRH377 pTargetVector-HR1+
pRH367 PacI x NotI ligated into pT.V. PacI x NotI

pRH378 pTargetVector-HR3+
pRH368 PacI x NotI ligated into pT.V. PacI x NotI

pRH379 pCDNA3.1-HA-mBCL9.2 (aa 2-556) **intermediate, HA not in frame**
PCR with mBCL9.2 f and mBCL9.2 556 r, ligated into pRH231 BamHI x XbaI

pRH380 pCDNA3.1-FLAG-mBCL9.2 (aa 1-556)
pRH379 BamHI x XbaI ligated into p85 BamHI x XbaI

pRH381 pBS-attB-UAS-Arm^{S10}(myc)-wt
pDA720 Asp718 x XbaI ligated into pBS-attB-UAS Asp718 x XbaI

pRH382 pBS-attB-UAS-Arm^{S10}(myc)-Y150A
pRH192 Asp718 x XbaI ligated into pBS-attB-UAS Asp718 x XbaI

pRH383 pBS-attB-UAS-Arm^{S10}(myc)-D172A
pDA722 Asp718 x XbaI ligated into pBS-attB-UAS Asp718 x XbaI

pRH384 pGAD424-Arm(myc)-Y150A (aa 38-806)
pRH191 PinAI x StuI ligated into pRH102 PinAI x StuI

pRH385 pBTM116-dα-catenin (aa 1-394)
pRH126 EcoRI x SalI ligated into pBTM116 EcoRI x SalI

pRH386 pBS-attB-tub-prom
pOP118 EcoRI x StuI blunt ligated into pBS-attB-UAS BamHI blunt

pRH387 pBS-attB-tub-Arm(myc)-wt
pDA716 Asp718 x XbaI ligated into pRH386 Asp718 x XbaI

pRH388 pBS-attB-tub-Arm(myc)-Y150A
pRH191 Asp718 x XbaI ligated into pRH386 Asp718 x XbaI

pRH389 pBS-attB-tub-Arm(myc)-D172A
pDA725 Asp718 x XbaI ligated into pRH386 Asp718 x XbaI

pRH390 pPacPL-FLAG-m/hβ-cat-wt
pRH95 BamHI x SalI ligated into pRH330 BamHI x XhoI

pRH391 pPacPL-FLAG-m/hβ-cat-Y142A
pRH137 BamHI x SalI ligated into pRH330 BamHI x XhoI

pRH392 pPacPL-FLAG-m/hβ-cat-D164A
pRH99 BamHI x SalI ligated into pRH330 BamHI x XhoI

pRH393 pPacPL-hP300-HA
pRH326 HindIII blunt x NotI ligated into pRH299 HpaI x NotI

pRH394 pTV-HR3-HR4 (LoxP-FRT-neo-FRT)
pRH361 AscI x XmaI and pRH362 PmeI x XmaI ligated into pRH360 AscI x PmeI

pRH395 pBS-attB-tub-FLAG-mBCL9.2-wt
pRH358 PacI x XbaI ligated into pRH386 PacI x XbaI

pRH396 pBS-attB-tub-FLAG-mBCL9.2-ΔPyBD
pRH359 PacI x XbaI ligated into pRH386 PacI x XbaI

pRH397 pBS-attB-tub-prom-3'UTR
pMZ55 EcoRI x XbaI blunt ligated into pRH386 XhoI blunt

pRH398 pTV.1-HR3-HR4 (FRT-neo-FRT)
pRH353 EcoRI x NotI blunt ligated into pRH394 SacII blunt

pRH399 pcDNA3.1-NLS-G4DBD
oligo BglII HA BamHI ligated into pRH307 BglII x BamHI

pRH401 pPacPL-FLAG-mBCL9.2ΔC (aa 1-556)
pRH380 NheI x XbaI ligated into pRH299 NheI

pRH402 pcDNA3.1-NLS-G4DBD-HA-mβ-cat-wt
pRH399 Asp718 x BamHI ligated into pRH194 Asp718 x BamHI

pRH403 pcDNA3.1-NLS-G4DBD-HA-mβ-cat-D164A
pRH399 Asp718 x BamHI ligated into pRH199 Asp718 x BamHI

pRH404 pcDNA3.1-NLS-G4DBD-HA-mβ-cat-ΔC
pRH399 Asp718 x BamHI ligated into pRH275 Asp718 x BamHI

pRH405 pcDNA3.1-NLS-G4DBD-HA-mβ-cat-D164A-ΔC
pRH399 Asp718 x BamHI ligated into pRH276 Asp718 x BamHI

pRH406 pcDNA3.1-NLS-G4DBD-HA-mβ-cat-Y142A
pRH399 Asp718 x BamHI ligated into pRH205 Asp718 x BamHI

pRH409 pBS-loxP-Hyg-loxP-HR2
loxP-Hyg-loxP rev (L-PGK-Hyg#12) ClaI blunt x NotI ligated into pRH341 SmaI x NotI

pRH410 pBS-HR1-loxP-Hyg-loxP-HR2-TK
pRH409 NotI x PmeI ligated into pRH340 NotI x PmeI

pRH411 pBS-HR1+-loxP-Hyg-loxP-HR2-TK
pRH409 NotI x PmeI ligated into pRH377 NotI x PmeI

pRH412 pcDNA3.1-NLS-VP16act-mCBP
p76 BamHI ligated into pRE629 BamHI

pRH413 pcDNA3.1-HA-mBCL9.2-FL
oligo NheI HA BamHI ligated into p85 NheI x BamHI

pRH414 pCS2-mb-catΔC-wt
PCR with SpeI mb-cat 536f x RI mb-catΔC r ligated into p95 SpeI x ClaI

pRH415 pCS2-mb-catΔC-D164A
PCR with SpeI mb-cat 536f x RI mb-catΔC r ligated into p96 SpeI x ClaI

pRH416 pAAV-mb-catΔC-wt
pRH414 StuI ligated into p97 StuI

pRH417 pAAV-mb-catΔC-D164A
pRH414 StuI ligated into p98 StuI

pRH418 pUAST-Arm-D164A
p47 Asp718 x XbaI ligated into pUAST Asp718 x XbaI

3.2 Constructs gifts

p1 pGAD424-hβ-cat 3-C'WT' (aa 196-781) cut out BamHI/SalI (von Kries, Berlin)
p2 pGAD424-hβ-cat WT-R (aa 196-781) cut out BamHI/SalI (von Kries, Berlin)
p3 pGAD424-hβ-cat R225A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p4 pGAD424-hβ-cat F253A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p5 pGAD424-hβ-cat H260A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p6 pGAD424-hβ-cat K270A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p7 pGAD424-hβ-cat R274A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p8 pGAD424-hβ-cat K292A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p9 pGAD424-hβ-cat W338A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p10 pGAD424-hβ-cat R342A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p11 pGAD424-hβ-cat K345A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p12 pGAD424-hβ-cat K354A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p13 pGAD424-hβ-cat R376A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p14 pGAD424-hβ-cat W383A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin**)**
p15 pGAD424-hβ-cat R386A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p16 pGAD424-hβ-cat K394A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p17 pGAD424-hβ-cat K435A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p18 pGAD424-hβ-cat R457A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p19 pGAD424-hβ-cat R469A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p20 pGAD424-hβ-cat H470A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p21 pGAD424-hβ-cat W504A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p22 pGAD424-hβ-cat N516A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p23 pGAD424-hβ-cat L427E (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p24 pGAD424-hβ-cat E462A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p25 pGAD424-hβ-cat N426A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p26 pGAD424-hβ-cat N430A (aa 216-781) cut out BamHI/SalI (von Kries, Berlin)
p27 pBTM116-mAPC (aa 1152-1393) (von Kries, Berlin)
p28 pBTM116-mE-cadherin (last 111 aa C-terminus) (von Kries, Berlin)
p29 pBS-mα-cat (Kemler, Freiburg)
p30 pCDNA3-mβ-cat (w/o KOZAK Barbara, TGC)
p31 pCDNA-ΔN-β-cat (aa 132-781) (Max, TGC)
p32 pBS-Arm^{S10} (George)
p33 pKB342 pBS + tubulin 3' UTR (George)
p34 pTK264 pUAS-ΔNarm-wt (Thomas)
p35 pTK265 pUAS-ΔNarm-E171A (Thomas)
p36 pTK266 pUAS-ΔNarm-D172A (Thomas)
p37 pTK267 pBS-ΔNarm-A303W/I304W (Thomas)
p38 pTK269 pUAS-ΔNarm-K443E (Thomas)
p39 pDA703 pBS-arm-wt (Denise)
p40 pDA704 pBS-arm-E171A (Denise)
p41 pDA705 pBS-arm-D172A (Denise)
p42 pDA734 pBS-arm-A303W/I304W (Denise)
p43 pDA707 pBS-arm-K443E (Denise)
p44 pDA715 tub-(myc)arm-E171A (Denise)
p45 pDA716 tub-(myc)arm-wt (Denise)
p46 pDA719 tub-(myc)arm-K443E (Denise)
p47 pDA725 tub-(myc)arm-D172A (Denise)
p48 pDA736 tub-(myc)arm-A303W/I304W (Denise)
p49 pTK293 tub-myr-(HA)-arm-wt (Thomas)
p50 pTK294 tub-myr-(HA)-arm-E171A (Thomas)
p51 pTK299 tub-myr-(HA)-arm-D172A (Thomas)
p52 pTK347 tub-myr-(HA)-arm-A303W/I304W (Thomas)
p53 pTK348 tub-myr-(HA)-arm-K443E (Thomas)
p54 pCMV-Lef (Liang)

p55 pCDNA-BCL9 (Barbara, TGC)
p56 pCDNA-hPygo1 (Barbara, TGC)
p57 pCDNA-hPygo2 (Barbara, TGC)
p59 pPGS-CMV-S33Y-hβ-cat (Eliane Müller, Bern)
p60 pCDNA-ΔN-ß-cat (aa 132-781) (Max, TGC) new
p61 pBS-hDvl2 (Sussman, Baltimore)
p62 pSVK3-hDvl2 (Sussman, Baltimore)
p63 pGAD-Ketel
p64 pBS-Ketel
p65 XBC-HA (Kimelman, Seattle)
p66 pBS-hPG (Eliane Müller, Bern)
p68 pPGS-ΔNTCF (Fearon, Ann Arbor)
p69 OT2-dα-cat (EST)
p70 M50 Super8xTOPflash (Moon, Seattle)
p71 M51 Super8xFOPflash (Moon, Seattle)
p72 pAc-LacZ
p73 pMT/V5/HisB-Gal4DB-Pygo (Reto pRE145)
p74 pMT/V5/HisB-Pygo (Reto pRE147)
p75 pGEM3Zf-mWnt3a (Nusse, Stanford)
p76 pCDNA3-mCBP-HA (Eckner, Newark)
p77 CMVß-hP300-wt (Eckner, Newark)
p78 CMVß-hP300-HA (Eckner, Newark)
p79 pBS-hLgs2 (HD1+HD2) (Pierre, TGC)
p80 pBS-dCBP (Smolik, Portland)
p81 pBS-Arm (George)
p82 pCDNA3-Arm (George)
p83 pGEX-4T-1-GST-hP300-CH3 (aa 1710-1891) (Weis, Stanford)
p84 pGEX-4T-3-GST-hICAT (Weis, Stanford)
p85 pCDNA3.1-FLAG-mBCL9.2-FL (Birchmeier, Berlin)
p86 pCDNA3.1-FLAG-mBCL9.2-Δ138-173 (Birchmeier, Berlin)
p87 pCDNA3.1-FLAG-mBCL9.2-ΔPyBD (Birchmeier, Berlin)
p88 pDA720 pUAST-ArmS10(myc)-wt (Denise)
p89 pDA721 pUAST-ArmS10(myc)-E171A (Denise)
p90 pDA722 pUAST-ArmS10(myc)-D172A (Denise)
p91 pDA737 pUAST-ArmS10(myc)-A303W/I304W (Denise)
p92 pDA724 pUAST-ArmS10(myc)-K443E (Denise)
p93 L-PGK-Hyg#12 (loxP-Hyg-loxP rev) (Shmerling, Polygene)
p94 pTargetVector pBS-FRT-neo-FRT-TK (Aguet, Lausanne)
p95 pCS2-mβ-cat(6 x myc)-wt (Kemler, Freiburg)
p96 pCS2-mβ-cat(6 x myc)-D164A (Kemler, Freiburg)
p97 pAAV-mβ-cat(6 x myc)-wt (Kemler, Freiburg)
p98 pAAV-mβ-cat(6 x myc)-D164A (Kemler, Freiburg)
p99 pDU10 (ESBATech, Zurich)
p100 pDU12 (ESBATech, Zurich)
p101 pDU13 (ESBATech, Zurich)
p102 pDU46 (ESBATech, Zurich)
p103 pDU62 (ESBATech, Zurich)
p104 pDU63 (ESBATech, Zurich)
p105 pDU106 (ESBATech, Zurich)
p106 pDU153 (ESBATech, Zurich)
p107 pDU198 (ESBATech, Zurich)

3.3 Oligonucleotides

Apa HR2b r	caccagggccctgcctag
Apa HR2c f	ctaggcagggccctggtg
Arm Y150Af1	ggtcaatctgatcaacgcccaggacgacgctgagc
Arm Y150Ar1	gctcagcgtcgtcctgggcgttgatcagattgacc
Asp718 Pme HR2c r	gctaggtaccgtttaaacccataatgaaggcgaacggc
Bam f mbcat	atggatccaccaccatggctactcaagctgacctg
Bam hP300 900f	gtacggatccaacatgggtcaacagccagc
Bam hP300 f	tagcggatccatggccgagaatgtggtgg
Bam mbcat	atggatccatgttgaaacatgcagttgtc
BCL9 199 Xba f	attctagaatgactatcgtctctttccacatc
BCL9 392 Not r	tagcggccgccaggattctgctgcggtcc
BCL9 732 S. NheI	atgctagcggccgctcacatctgagagttggatcccatg
BglII K. BCL9	atagatctaccaccatgcattccagtaaccctaaag
D162A f mbcatD164A	gctgacaaaactgctaaacgctgaggcccaggtggtag
D162A r mbcatD164A	ctaccacctgggcctcagcgtttagcagttttgtcagc
da-cat EcoRI	atgaattcatgttaaaacctgataaaatggg
da-cat SalI	atgtcgacgaaacatgatccacaacagcc
dE-cad (EcoRI)	gcgaattcgcagtggtggtgcagaaaaagc
Eco Asc HR2a f1	tagcgaattcggcgcgcccagtgatgtaacttgcttttcag
EcoRI HR4a r	ctgagaattcttaccgcttcttgtaatcctgtgg
EcoRI HR4b f	tcaggaattcccagctaccgttcttttcac
EcoRI PacI HR3 f.1	tacggaattcttaattaagttgtgggttagatcttgcatgc
EcoRI PacI HR3f test	tacggaattcttaattaaggtgaactgttagcagggagc
Gal4BD Asp718 f	atggtaccaccatgaagctactgtcttctatcg
Gal4BD Bam r	taggatcccgatacagtcaactgtctttg
hbcat D164Af	ctgctaaatgacgaggcccaggtggtggttaataag
hbcat D164Ar	cttattaaccaccacctgggcctcgtcatttagcag
hbcat E163Af	caaaactgctaaatgacgcggaccaggtggtgg
hbcat E163Ar	ccaccacctggtccgcgtcatttagcagttttg
hbcat R386Af	ctgtctttggactctcgcgaatctttcagatgctg
hbcat R386Ar	cagcatctgaaagattcgcgagagtccaaagacag
hbcat-XbaI	agtctagattacaggtcagtatcaaaccagg
hLgs2 HD1+2f	atgaattctcgcagttcgtatatgtcttc
hLgs2 HD1+2r	atgcggccgcttgaggaagggctcagtctc
hP300 CH3f	atggatccttctcttcactccgaagagc
hP300 CH3r	atgcggccgcatctggtgttggattggc
hTCF4 Not r	tagcggccgctctggagataggttcgggc

hTCF4 Xba f	attctagaatgccgcagctgaacggcgg
m.m.Lef EcoRI	tcgaattccaccatgccccaactttccggagg
m.m.Lef XbaI	agtctagaatgtaggcagctgtcattctgg
mbcat D164A f	ctgctaaacgatgaggcccaggtggtagttaataaagc
mbcat D164A r	gctttattaactaccacctgggcctcatcgtttagcag
mbcat E664Af	ctattccgaatgtctgcggacaagccacaggattac
mbcat E664Ar	gtaatcctgtggcttgtccgcagacattcggaatag
mbcat F660A on E664Af	gcagctgctgtcctagcccgaatgtctgcggac
mbcat F660A on E664Ar	gtccgcagacattcgggctaggacagcagctgc
mbcat F660Af	gcagctgctgtcctagcccgaatgtctgaggac
mbcat F660Ar	gtcctcagacattcgggctaggacagcagctgc
mbcat R661A on F660Af	gctgctgtcctagccgcaatgtctgaggacaag
mbcat R661A on F660Ar	cttgtcctcagacattgcggctaggacagcagc
mbcat R661Af	gctgctgtcctattcgcaatgtctgaggacaag
mbcat R661Ar	cttgtcctcagacattgcgaataggacagcagc
mbcat XhoIr	tcagctcgagtacaggtcagtatcaaaccagg
mbcat Y142Af	gttgtcaatttgattaacgctcaggatgacgcggaac
mbcat Y142Ar	gttccgcgtcatcctgagcgttaatcaaattgacaac
mbcat Y142Df	gttgtcaatttgattaacgatcaggatgacgcggaac
mbcat Y142Dr	gttccgcgtcatcctgatcgttaatcaaattgacaac
mbcat Y142Ef	gttgtcaatttgattaacgaacaggatgacgcggaac
mbcat Y142Er	gttccgcgtcatcctgttcgttaatcaaattgacaac
mbcat Y142Ff	gttgtcaatttgattaactttcaggatgacgcggaac
mbcat Y142Fr	gttccgcgtcatcctgaaagttaatcaaattgacaac
mbcat Y654Af	gaaggcgtggcaacagccgcagctgctgtcctattc
mbcat Y654Ar	gaataggacagcagctgcggctgttgccacgccttc
mbcat Y654Ef	gaaggcgtggcaacagaagcagctgctgtcctattc
mbcat Y654Er	gaataggacagcagctgcttctgttgccacgccttc
mbcatΔC NotI	atgcggccgctccgcttcttgtaatcctgtgg
mbcat-Asp718	caggtaccaccatggctactcaagctgacctg
mb-catR1-f	atgaattcggatccaactatcaggatgacgcggaac
mb-catR1-Y142A f	atgaattcggatccaacgctcaggatgacgcggaac
mb-catR4-r	atgcggccgcataagctaagatctgaaggcag
mBCL9.2 556 r	cgtatctagattccgcggcccatcatacccccccattcc
mBCL9.2 f	ggccgctgaggatcctgg
mCBP 316 NotI r	atgcggccgccggtaccagtcattcccatc
mCBP XbaI f	attctagaatggccgagaacttgctgg
mWnt3a f	agaagcttaccaccatggctcctctcggatacctc
mWnt3a r	ctgaattcaatctagactcttgcaggtgtgcacgtcatag
Not (mut) HR1 r1	ctgagcggccgcctgcttacctgggcctcatc

Not HR3 r.1	cgtagcggccgctcctcagcttacccacgcc
Not Pme HR4d r	cgtagcggccgcgtttaaacggtcatatggttggcctagg
RI mbcatΔC r	gcatgaattcatcgatgggatccccgcttcttgtaatcctgtgg
Sal Asc HR4a f	tcaggtcgacggcgcgccgagcccagtcccattagttg
SpeI mbcat 536 f	gctattccacgactagttcag
Spe Pac HR1 f	tcagactagttaattaaggagatgttacactattgaatc
Spe Pac HR1f test	tcagactagttaattaagtgtgtcactttgaactgtgg
Xba hP300 r	tcagtctagagtagcaagacttgcctgactgg
XbaI HR4c r.1	gatctctctctagagaacag
XbaI HR4d f	gtctgttctctagagagagatc
Xho HR2a r	caccacctcgagctagagc
Xho HR2b f	gctctagctcgaggtggtg
XhoI GFP f	ctcgagcgtgagcaagggcgaggagc
XmaI HR4b r	cagacagcccgggtacccc
XmaI HR4c f.1	gggtacccgggctgtctgg

3.4 Fly Stocks

Abbreviations
'X=' = homozygous on X

RH1 *arm*^{2a9}/Bin ; +/+ ; tub-Arm(myc)-D172A Prd-Gal4/TM3 ; +/+
tub-arm-D172A derived from pDA725,2

RH2 *arm*^{2a9}/Bin ; +/+ ; tub-arm-wt Prd-Gal4/TM3 ; +/+
716,4

RH3 *y w* UAS-RFP/X= ; UAS-Arm^{S10}(myc)-D172A UAS-GFP/Cyo ; +/+ ; +/+
722,2

RH4 *y w* UAS-RFP/X= ; UAS-ΔNarm-wt UAS-GFP/Cyo ; +/+ ; +/+

RH5 *y w* UAS-RFP/X= ; UAS-Arm^{S10}(myc)-wt UAS-GFP/Cyo ; +/+ ; +/+

RH6 *y w* UAS-RFP/X= ; Sp/Cyo ; +/+ ; +/+

RH7 *w* hs-flp hs-GFP FRT18/X= ; +/+ ; tub-Arm-wt/3= ; +/+
716,4

RH8 *w* hs-flp hs-GFP FRT18/X= ; +/+ ; tub-Arm-D172A/TM6b ; +/+
725,2

RH9 *w* hs-flp hs-GFP FRT18/X= ; +/+ ; TM6b/MKRS ; +/+

RH10 *y w* hs-flp/X= ; UAS-ΔNarm-wt UAS-GFP/2= ; +/+ ; +/+

RH11 *y w* hs-flp/X= ; UAS-arm^{S10}(myc)-wt UAS-GFP/2= ; +/+ ; +/+

RH12 *y w* hs-flp/X= ; UAS-arm^{S10}(myc)-D172A UAS-GFP/Cyo ; +/+ ; +/+
722,2

RH13 *w* hs-flp hs-GFP FRT18/X= ; +/+ ; tub-myr-Arm-wt/TM6b ; +/+
753,5

RH14a *y w* hs-flp/X= ; tub-Arm(myc)-wt (716,1)/Cyo ; +/+ ; +/+
Derived from pDA716 (p45)

RH14b *y w* hs-flp/X= ; tub-Arm(myc)-wt (716,2)/Cyo ; +/+ ; +/+
Derived from pDA716 (p45)

RH14c *y w* hs-flp/X= ; +/+ ; tub-Arm(myc)-wt (716,4)/TM6b ; +/+
Derived from pDA716 (p45)

RH14d *y w* hs-flp/X= ; +/+ ; tub-Arm(myc)-wt (716,6)/TM6b ; +/+
Derived from pDA716 (p45)

RH15a *y w* hs-flp/X= ; +/+ ; tub-Arm(myc)-E171A (715,1)/TM6b ; +/+
Derived from pDA715 (p44)

RH15b *y w* hs-flp/X= ; +/+ ; tub-Arm(myc)-E171A (715,2)/TM6b ; +/+
Derived from pDA715 (p44)

RH15c *y w* hs-flp/X= ; tub-Arm(myc)-E171A (715,3)/Cyo ; +/+ ; +/+
Derived from pDA715 (p44)

RH15d *y w* hs-flp/X= ; tub-Arm(myc)-E171A (715,6)/Cyo ; +/+ ; +/+
Derived from pDA715 (p44)

RH16a *y w* hs-flp/X= ; tub-Arm(myc)-D172A (725,1)/Cyo ; +/+ ; +/+
Derived from pDA725 (p47)

RH16b *y w* hs-flp/X= ; +/+ ; tub-Arm(myc)-D172A (725,2)/TM6b ; +/+
Derived from pDA725 (p47)

RH16c *y w* hs-flp/X= ; +/+ ; tub-Arm(myc)-D172A (725,3)/3= ; +/+
Derived from pDA725 (p47)

RH16d *y w* hs-flp/X= ; tub-Arm(myc)-D172A (725,4)/Cyo ; +/+ ; +/+
Derived from pDA725 (p47)

RH17a *y w* hs-flp/X= ; tub-Arm(myc)-K443E (719,1)/Cyo ; +/+ ; +/+
Derived from pDA719 (p46)

RH17b *y w* hs-flp/X= ; tub-Arm(myc)-K443E (719,3)/Cyo ; +/+ ; +/+
Derived from pDA719 (p46)

RH17c *y w* hs-flp/X= ; tub-Arm(myc)-K443E (719,4)/Cyo ; +/+ ; +/+
Derived from pDA719 (p46)

RH18a *y w* hs-flp/X= ; +/+ ; UAS-ΔNArm-wt (264,1)/3=; +/+
Derived from pTK264 (p34)

RH18b UAS-ΔNArm-wt (264,2)/X= ; +/+ ; +/+ ; +/+
Derived from pTK264 (p34)

RH18c *y w* hs-flp/X= ; +/+ ; UAS-ΔNArm-wt (264,3)/3= ; +/+
Derived from pTK264 (p34)

RH19a *y w* hs-flp/X= ; +/+ ; UAS-ΔNArm-E171A (265,1)/ TM6b ; +/+
Derived from pTK265 (p35)

RH19b *y w* hs-flp/X= ; +/+ ; UAS-ΔNArm-E171A (265,3)/TM6b ; +/+
Derived from pTK265 (p35)

RH19c *y w* hs-flp/X= ; UAS-ΔNArm-E171A (265,9)/Cyo ; +/+ ; +/+
Derived from pTK265 (p35)

RH20a *y w* hs-flp/X= ; +/+ ; UAS-ΔNArm-D172A (266,1)/TM6b ; +/+
Derived from pTK266 (p36)

RH20b *y w* hs-flp/X= ; +/+ ; UAS-ΔNArm-D172A (266,3)/3= ; +/+
Derived from pTK266 (p36)

RH20c *y w* hs-flp/X= ; UAS-ΔNArm-D172A (266,7)/Cyo ; +/+ ; +/+
Derived from pTK266 (p36)

RH21a *y w* hs-flp/X= ; UAS-ΔNArm-K443E (269,1)/2= ; +/+ ; +/+
Derived from pTK269 (p38)

RH21b *y w* hs-flp/X= ; UAS-ΔNArm-K443E (269,3)/2= ; +/+ ; +/+
Derived from pTK269 (p38)

RH21c *y w* hs-flp/X= ; +/+ ; UAS-ΔNArm-K443E (269,4)/TM6b ; +/+
Derived from pTK269 (p38)

RH22a *y w* hs-flp/X= ; UAS-ΔNArm-A303W/I304W (270,1)/Cyo ; +/+ ; +/+
Derived from pTK270

RH22b *y w* hs-flp/X= ; UAS-ΔNArm-A303W/I304W (270,2)/Cyo ; +/+ ; +/+
Derived from pTK270

RH22c *y w* hs-flp/X= ; +/+ ; UAS-ΔNArm-A303W/I304W (270,3)/TM6b ; +/+
Derived from pTK270

RH23a *y w* hs-flp/X= ; UAS-Arm^{S10}-wt (720,2)/Cyo ; +/+ ; +/+
Derived from pDA720 (p88)

RH23b *y w* hs-flp/X= ; +/+ ; UAS-Arm^{S10}-wt (720,4)/TM6b ; +/+
Derived from pDA720 (p88)

RH23c *y w* hs-flp/X= ; +/+ ; UAS-Arm^{S10}-wt (720,6)/TM6b ; +/+
Derived from pDA720 (p88)

RH24a *y w* hs-flp/X= ; +/+ ; UAS-Arm^{S10}-E171A (721,1)/TM6b ; +/+
Derived from pDA721 (p89)

RH24b *y w* hs-flp/X= ; +/+ ; UAS-Arm^{S10}-E171A (721,2)/TM6b ; +/+
Derived from pDA721 (p89)

RH24c *y w* hs-flp/X= ; UAS-Arm^{S10}-E171A (721,3)/Cyo ; +/+ ; +/+
Derived from pDA721 (p89)

RH25a *y w* hs-flp/X= ; +/+ ; UAS-Arm^{S10}-D172A (722,1)/TM6b ; +/+
Derived from pDA722 (p90)

RH25b *y w* hs-flp/X= ; UAS-Arm^{S10}-D172A (722,2)/Cyo ; +/+ ; +/+
Derived from pDA722 (p90)

RH25c *y w* hs-flp/X= ; UAS-Arm^{S10}-D172A (722,3)/Cyo ; +/+ ; +/+
Derived from pDA722 (p90)

RH26a *y w* hs-flp/X= ; +/+ ; UAS-Arm^{S10}-K443E (724,1)/3= ; +/+
Derived from pDA724 (p92)

RH26b *y w* hs-flp/X= ; +/+ ; UAS-Arm^{S10}- K443E (724,2)/TM6b ; +/+
Derived from pDA724 (p92)

RH26c *y w* hs-flp/X= ; UAS-Arm^{S10}- K443E (724,3)/Cyo ; +/+ ; +/+
Derived from pDA724 (p92)

RH27a *y w* hs-flp/X= ; UAS-Arm^{S10}- A303W/I304W (737,1)/2= ; +/+ ; +/+
Derived from pDA737 (p91)

RH27b *y w* hs-flp/X= ; +/+ ; UAS-Arm^{S10}- A303W/I304W (737,2)/3= ; +/+
Derived from pDA737 (p91)

RH27c *y w* hs-flp/X= ; +/+ ; UAS-Arm^{S10}- A303W/I304W (737,5)/TM6b ; +/+
Derived from pDA737 (p91)

RH28a *y w* hs-flp/X= ; +/+ ; tub-myr-Arm-wt (753,2)/TM6b ; +/+
Derived from pDA753

RH28b *y w* hs-flp/X= ; +/+ ; tub-myr-Arm-wt (753,5)/TM6b ; +/+
Derived from pDA753

RH28c *y w* hs-flp/X= ; tub-myr-Arm-wt (753,7)/Cyo ; +/+ ; +/+
Derived from pDA753

RH29a *y w* hs-flp/X= ; +/+ ; tub-myr-Arm-E171A (754,2)/TM6b ; +/+
Derived from pDA754

RH29b *y w* hs-flp/X= ; +/+ ; tub-myr-Arm-E171A (754,3)/TM6b ; +/+
Derived from pDA754

RH29c *y w* hs-flp/X= ; tub-myr-Arm-E171A (754,4)/Cyo ; +/+ ; +/+
Derived from pDA754

RH29d *y w* hs-flp/X= ; +/+ ; tub-myr-Arm-E171A (754,6)/TM6b ; +/+
Derived from pDA754

RH30 *y w* hs-flp/X= ; tub-myr-Arm-D172A (755,1)/Cyo ; +/+ ; +/+
Derived from pDA755

RH31a *y w* hs-flp/X= ; +/+ ; tub-myr-Arm-K443E (757,2)/TM6b ; +/+
Derived from pDA757

RH31b *y w* hs-flp/X= ; +/+ ; tub-myr-Arm-K443E (757,3)/TM6b ; +/+
Derived from pDA757

RH32a *y w* hs-flp/X= ; +/+ ; tub-NLS-HA-dLgsΔHD1 (201,3)/TM6b ; Dp1021 [y+] spa[pol]/4=
Derived from pRH201

RH32b *y w* hs-flp/X= ; +/+ ; tub-NLS-HA-dLgsΔHD1 (201,4)/TM6b ; Dp1021 [y+] spa[pol]/4=
Derived from pRH201

RH32c *y w* hs-flp/X= ; +/+ ; tub-NLS-HA-dLgsΔHD1 (201,7)/TM6b ; Dp1021 [y+] spa[pol]/4=
Derived from pRH201

RH32d *y w* hs-flp/X= ; +/+ ; tub-NLS-HA-dLgsΔHD1 (201,8)/TM6b ; Dp1021 [y+] spa[pol]/4=
Derived from pRH201

RH33a *y w* hs-flp/X= ; tub-Arm(myc)-Y150A (191,1)/Cyo ; +/+ ; +/+
Derived from pRH191

RH33b tub-Arm(myc)-Y150A (191,2)/FM7 ; +/+ ; +/+ ; +/+
Derived from pRH191

RH33c *y w* hs-flp/X= ; +/+ ; tub-Arm(myc)-Y150A (191,4)/TM6b ; +/+
Derived from pRH191

RH33d *y w* hs-flp/X= ; +/+ ; tub-Arm(myc)-Y150A (191,5)/TM6b ; +/+
Derived from pRH191

RH33e *y w* hs-flp/X= ; tub-Arm(myc)-Y150A (191,9)/Cyo ; +/+ ; +/+
Derived from pRH191

RH34a *y w* hs-flp/X= ; +/+ ; UAS-Arm(myc) ^{S10}-Y150A (192,1)/3= ; +/+
Derived from pRH192

RH34b *y w* hs-flp/X= ; UAS-Arm(myc) ^{S10}-Y150A (192,2)/Cyo ; +/+ ; +/+
Derived from pRH192

RH34c *y w* hs-flp/X= ; +/+ ; UAS-Arm(myc)^{S10}-Y150A (192,3)/3= ; +/+
Derived from pRH192

RH34d *y w* hs-flp/X= ; UAS-Arm(myc) ^{S10}-Y150A (192,4)/Cyo ; +/+ ; +/+
Derived from pRH192

RH34e *y w* hs-flp/X= ; UAS-Arm(myc) ^{S10}-Y150A (192,5)/Cyo ; +/+ ; +/+
Derived from pRH192

RH35 *w* hs-flp hs-GFP FRT18/X= ; +/+ ; tub-Arm(myc)-Y150A (191,4)/TM6b ; +/+
Derived from RH33c

RH36a tub-Arm(myc)-ΔC (310,1)/FM7 ; +/+ ; +/+ ; +/+
Derived from pRH310

RH36b *y w* hs-flp/X= ; tub-Arm(myc)-ΔC (310,2)/Cyo ; +/+ ; +/+
Derived from pRH310

RH36c *y w* hs-flp/X= ; +/+ ; tub-Arm(myc)-ΔC (310,3)/TM6b ; +/+
Derived from pRH310

RH36d *y w* hs-flp/X= ; tub-Arm(myc)-ΔC (310,4)/Cyo ; +/+ ; +/+
Derived from pRH310

RH36e *y w* hs-flp/X= ; tub-Arm(myc)-ΔC (310,5)/Cyo ; +/+ ; +/+
Derived from pRH310

RH36f *y w* hs-flp/X= ; +/+ ; tub-Arm(myc)-ΔC (310,6)/TM6b ; +/+
Derived from pRH310

RH37 *y w* hs-flp/X= ; +/+ ; tub-Arm(myc)-D172A-ΔC (311,1)/TM6b ; +/+
Derived from pRH311

RH38 *w* hs-flp hs-GFP FRT18/X= ; +/+ ; tub-Arm(myc)-ΔC (310,3)/TM6b ; +/+
310,3

RH39 *w* hs-flp hs-GFP FRT18/X= ; +/+ ; tub-Arm(myc)-D172A-ΔC (311,1)/TM6b ; +/+
311,1

RH40 *y w* hs-flp/X= ; +/+ ; tub-BCL9.2 (358,1)/TM6b ; Dp1021 [y+] spa[pol]/4=
Derived from pRH358

RH41 *y w* hs-flp/X= ; +/+ ; tub-BCL9.2 (395,1)/TM6b ; Dp1021 [y+] spa[pol]/4=
Derived from pRH395 with attB system

RH42 *y w* hs-flp/X= ; +/+ ; tub-BCL9.2 (396,1)/TM6b ; Dp1021 [y+] spa[pol]/4=
Derived from pRH396 with attB system

RH43 *y w* hs-flp/X= ; +/+ ; UAS-ArmS10(myc)-D172A (383,1)/TM6b ; +/+
Derived from pRH383 with attB system

RH44 *y w* hs-flp/X= ; +/+ ; tub-Arm(myc)-wt (387,1)/TM6b ; +/+
Derived from pRH387 with attB system

RH45 *y w* hs-flp/X= ; +/+ ; tub-Arm(myc)-Y150A (388,1)/TM6b ; +/+
Derived from pRH388 with attB system

RH46 Daughterless-Gal4/3=

3.5 References

Adachi, S., Jigami, T., Yasui, T., Nakano, T., Ohwada, S., Omori, Y., Sugano, S., Ohkawara, B., Shibuya, H., Nakamura, T. et al. (2004). Role of a BCL9-related beta-catenin-binding protein, B9L, in tumorigenesis induced by aberrant activation of Wnt signaling. *Cancer Res* **64**, 8496-501.

Barker, N., Hurlstone, A., Musisi, H., Miles, A., Bienz, M. and Clevers, H. (2001). The chromatin remodelling factor Brg-1 interacts with beta-catenin to promote target gene activation. *Embo J* **20**, 4935-43.

Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R. and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* **382**, 638-42.

Brembeck, F. H., Schwarz-Romond, T., Bakkers, J., Wilhelm, S., Hammerschmidt, M. and Birchmeier, W. (2004). Essential role of BCL9-2 in the switch between beta-catenin's adhesive and transcriptional functions. *Genes Dev* **18**, 2225-30.

Brunner, E., Peter, O., Schweizer, L. and Basler, K. (1997). pangolin encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in Drosophila. *Nature* **385**, 829-33.

Graham, T. A., Weaver, C., Mao, F., Kimelman, D. and Xu, W. (2000). Crystal structure of a beta-catenin/Tcf complex. *Cell* **103**, 885-96.

Hecht, A., Vleminckx, K., Stemmler, M. P., van Roy, F. and Kemler, R. (2000). The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. *Embo J* **19**, 1839-50.

Hoffmans, R. and Basler, K. (2004). Identification and in vivo role of the Armadillo-Legless interaction. *Development* **131**, 4393-400.

Hoffmans, R., Stadeli, R. and Basler, K. (2005). Pygopus and legless provide essential transcriptional coactivator functions to armadillo/beta-catenin. *Curr Biol* **15**, 1207-11.

Kramps, T., Peter, O., Brunner, E., Nellen, D., Froesch, B., Chatterjee, S., Murone, M., Zullig, S. and Basler, K. (2002). Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. *Cell* **109**, 47-60.

Orsulic, S. and Peifer, M. (1996). An in vivo structure-function study of armadillo, the beta-catenin homologue, reveals both separate and overlapping regions of the protein required for cell adhesion and for wingless signaling. *J Cell Biol* **134**, 1283-300.

Peifer, M., Orsulic, S., Sweeton, D. and Wieschaus, E. (1993). A role for the Drosophila segment polarity gene armadillo in cell adhesion and cytoskeletal integrity during oogenesis. *Development* **118**, 1191-207.

Peifer, M. and Wieschaus, E. (1990). The segment polarity gene armadillo encodes a functionally modular protein that is the Drosophila homolog of human plakoglobin. *Cell* **63**, 1167-76.

Stadeli, R. and Basler, K. (2005). Dissecting nuclear Wingless signalling: Recruitment of the transcriptional co-activator Pygopus by a chain of adaptor proteins. *Mech Dev* **122**, 1171-82.

Takemaru, K. I. and Moon, R. T. (2000). The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression. *J Cell Biol* **149**, 249-54.

Townsley, F. M., Cliffe, A. and Bienz, M. (2004). Pygopus and Legless target Armadillo/beta-catenin to the nucleus to enable its transcriptional co-activator function. *Nat Cell Biol* **6**, 626-33.

Tutter, A. V., Fryer, C. J. and Jones, K. A. (2001). Chromatin-specific regulation of LEF-1-beta-catenin transcription activation and inhibition in vitro. *Genes Dev* **15**, 3342-54.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A. et al. (1997). Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell* **88**, 789-99.

Lebenslauf

Name:	HOFFMANS
Vorname	Raymond
Geboren:	am 16. Januar 1977 in Apeldoorn, Die Niederlande
Nationalität:	Niederländisch

Ausbildung:

1989 - 1997	Atheneum, De Heemgaard Apeldoorn (NL)
1997 - 2002	MSc Biologie, Universität Utrecht (NL)
2000 - 2001	Diplomarbeit an der Universität Utrecht (NL) unter Leitung von Dr. Renze Heidstra und Prof. Dr. Ben Scheres: 'Characterization and isolation of three QC specific promotors'
2001 - 2002	Diplomarbeit am Institut für Molekularbiologie der Universität Zürich (CH) unter der Leitung von Prof. Dr. Konrad Basler: 'Fine mapping the interaction between β-catenin and BCL9'
2002 - 2006	Doktorand am Institut für Molekularbiologie der Universität Zürich (CH) unter der leitung von Prof. Dr. Konrad Basler: 'Characterization of the molecular functions of Armadillo, Legless and Pygopus in nuclear Wingless/Wnt signalling'

Publikationen:

Hoffmans R. and Basler K. (2004). Identification and in vivo role of the Armadillo-legless interaction. Development. 131, 4393-4400.

Hoffmans R.[1], Städeli R.[1] and Basler K. (2005). Pygopus and Legless provide essential transcriptional coactivator functions to Armadillo/β-catenin Curr Biol. 15, 1207-1211.

([1] These authors contributed equally to this work and should be considered co-first authors)

Acknowledgements

Foremost I would like to thank Koni for giving me the opportunity to do my PhD in his lab and for the many fruitful discussions I had with him during that time. Besides having a huge impact on my projects, he was also a major influence on the maturation of my scientific thinking.

I would especially like to thank Reto, with whom I had close and very fruitful collaborations. I really enjoyed working and writing together with him. I also would like to thank George and Christian. Together these three guys gave me invaluable advice, suggestions and comments on experiments and manuscripts.

I would also like to thank all the past and present members of the Basler lab for the good atmosphere and working conditions in the lab. I really enjoyed the scientific interactions and the non-scientific conversations in the lab. In particular I would like to thank Thomas for showing me how to work with yeast, Denise for introducing me to flies, George for introducing me to tissue culture and Alister for giving me the first working version of the wf-luciferase reporter construct. I also would like to thank all the people with whom I spent time in the 18ºC room and who helped me a lot with the injections (Denise, Chrigi, Chloe, Alister, Tamara, Reto, Peter, Johannes, Esther and Christian). One of the great things in lab is that everybody is very open and willing to help you when you run into problems.

Finally I would like to thank Tamara. For all her love and support during my PhD. Also for her critical remarks and constructive criticism on most of the work you (might) have read in this thesis.